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(54) Title: CHIMERIC FLAVIVIRUS VACCINES

(57) Abstract: A chimeric live, infectious, attenuated virus containing a yellow fever virus, in which the nucleotide sequence for a prM-E protein is either deleted, truncated, or mutated, so that functional prM-E protein is not expressed, and integrated into the genome of the yellow fever virus, a nucleotide sequence encoding a prM-E protein of a second, different flavivirus, so that the prM-E protein of the second flavivirus is expressed.

CHIMERIC FLAVIVIRUS VACCINES

Background of the Invention

This invention relates to infectious, attenuated viruses useful as vaccines against diseases caused by flaviviruses.

Several members of the flavivirus family pose current or potential threats to global public health. For example, Japanese encephalitis is a significant public health problem involving millions of at risk individuals in the Far East. Dengue virus, with an estimated annual incidence of 100 million cases of primary dengue fever and over 450,000 cases of dengue hemorrhagic fever worldwide, has emerged as the single most important arthropod-transmitted human disease.

Other flaviviruses continue to cause endemic diseases of variable nature and have the potential to emerge into new areas as a result of changes in climate, vector populations, and environmental disturbances caused by human activity. These flaviviruses include, for example, St. Louis encephalitis virus, which causes sporadic, but serious, acute disease in the midwest, southeast, and western United States; West Nile virus, which causes febrile illness, occasionally complicated by acute encephalitis, and is widely distributed throughout Africa, the Middle East, the former Soviet Union, and parts of Europe; Murray Valley encephalitis virus, which causes endemic nervous system disease in Australia; and Tick-borne encephalitis virus, which is distributed throughout the former Soviet Union and eastern Europe, where its *Ixodes* tick vector is prevalent and responsible for a serious form of encephalitis in those regions.

Hepatitis C virus (HCV) is another member of the flavivirus family, with a genome organization and replication strategy that are similar, but

E protein from the irreversible conformational changes caused by maturation in the acidic vesicles of the exocytic pathway (Guirakhoo *et al.*, Virology 191:921-931, 1992).

The cleavage of prM to M protein occurs shortly before release of virions by a furin-like cellular protease (Stadler *et al.*, J. Virol. 71:8475-8481, 1997), which is necessary to activate hemagglutinating activity, fusogenic activity, and infectivity of virions. The M protein is cleaved from its precursor protein (prM) after the consensus sequence R-X-R/K-R (X is variable), and incorporated into the virus lipid envelope together with the E protein.

Cleavage sequences have been conserved not only within flaviviruses, but also within proteins of other, unrelated viruses, such as PE2 of murine coronaviruses, PE2 of alphaviruses, HA of influenza viruses, and p160 of retroviruses. Cleavage of the precursor protein is essential for virus infectivity, but not particle formation. It was shown that, in case of a TBE-dengue 4 chimera, a change in the prM cleavage site resulted in decreased neurovirulence of this chimera (Pletnev *et al.*, J. Virol. 67:4956-4963, 1993), consistent with the previous observation that efficient processing of the prM is necessary for full infectivity (Guirakhoo *et al.*, 1991, *supra*; Guirakhoo *et al.*, 1992, *supra*; Heinz *et al.*, Virology 198:109-117, 1994). Antibodies to prM protein can mediate protective immunity, apparently due to neutralization of released virions that contain some uncleaved prM. The proteolytic cleavage site of the PE2 of VEE (4 amino acids) was deleted by site-directed mutagenesis of the infectious clone (Smith *et al.*, ASTMH meeting, December 7-11, 1997). Deletion mutants replicated with high efficiency and PE2 proteins were incorporated into particles. This mutant was evaluated in lethal mouse and

Philadelphia, 1995). In addition, the yellow fever virus has been studied at the genetic level (Rice *et al.*, Science 229:726-733, 1985) and information correlating genotype and phenotype has been established (Marchevsky *et al.*, Am. J. Trop. Med. Hyg. 52:75-80, 1995). Specific examples of yellow fever substrains that can be used in the invention include, for example, YF 17DD (GenBank Accession No. U17066), YF 17D-213 (GenBank Accession No. U17067), YF 17D-204 France (X15067, X15062), and YF-17D-204, 234 US (Rice *et al.*, Science 229:726-733, 1985; Rice *et al.*, New Biologist 1:285-296, 1989; C 03700, K 02749). Yellow Fever virus strains are also described by Galler *et al.*, Vaccine 16 (9/10):1024-28, 1998.

Preferred flaviviruses for use as the second flavivirus in the chimeric viruses of the invention, and thus sources of immunizing antigen, include Japanese Encephalitis (JE, *e.g.*, JE SA14-14-2), Dengue (DEN, *e.g.*, any of Dengue types 1-4; for example, Dengue-2 strain PUO-218) (Gruenberg *et al.*, J. Gen. Virol. 67:1391-1398, 1988) (sequence appendix 1; nucleotide sequence of Dengue-2 insert; Pr-M: nucleotides 1-273; M: nucleotides 274-498; E: nucleotides 499-1983) (sequence appendix 1; amino acid sequence of Dengue-2 insert; Pr-M: amino acids 1-91; M: amino acids 92-166; E: amino acids 167-661), Murray Valley Encephalitis (MVE), St. Louis Encephalitis (SLE), West Nile (WN), Tick-borne Encephalitis (TBE) (*i.e.*, Central European Encephalitis (CEE) and Russian Spring-Summer Encephalitis (RSSE) viruses), and Hepatitis C (HCV) viruses. Additional flaviviruses for use as the second flavivirus include Kunjin virus, Powassan virus, Kyasanur Forest Disease virus, and Omsk Hemorrhagic Fever virus. As is discussed further below, the second flavivirus sequences can be provided from two different second flaviviruses, such as two Dengue strains.

Fever 17D), the resulting chimeric virus is attenuated to a degree that renders it safe for use in humans.

Other features and advantages of the invention will be apparent from the following detailed description, the drawings, and the claims.

Brief Description of the Drawings

Fig. 1A is a schematic representation of processing events at the C/prM junction of parental viruses that can be used in the invention.

Fig. 1B is a schematic representation of the sequences in the capsid, prM signal, and prM regions of flaviviruses that can be used in the invention (SEQ ID NOs:54-70).

Fig. 2 is a schematic representation of the approach to making chimeric flaviviruses at the prM signal region used (SEQ ID NOs:71 and 72) by C.J. Lai (WO 93/06214).

Fig. 3 is a schematic representation of an attempt to use the method of C.J. Lai (WO 93/06214) with a yellow fever backbone (SEQ ID NOs:73 and 74).

Fig. 4 is a schematic representation illustrating that the viability of flavivirus chimeras depends on the choice of signal.

Fig. 5 is a schematic representation of the cloning method used in the present invention, at the prM signal region (SEQ ID NOs:75-77).

Fig. 6 is a schematic representation of the C, prM, E, and NS1 regions and junction sequences of a YF/JE chimera of the invention. The amino acid sequences flanking cleavage sites at the junctions are indicated for JE, YF, and the YF/JE chimera (SEQ ID NOs:78-85).

Fig. 7 is a schematic representation of genetic manipulation steps that were carried out to construct a Yellow-Fever/Japanese Encephalitis (YF/JE) chimeric virus of the invention.

Fig. 16 is a series of graphs showing the serological responses of mice immunized with a single dose of the live viruses indicated in the figure.

Fig. 17 is a set of graphs showing viremia and GMT of viremia in 3 rhesus monkeys inoculated with ChimeriVax or YF-Vax by the i.c. route.

Fig. 18 is a graph showing the PRNT neutralizing antibody titers (50%) in rhesus monkeys 2 and 4 weeks post inoculation with a single dose of YF-Vax or ChimeriVax vaccines by the i.c. route.

Fig. 19 is a graph showing the results of neurovirulence testing of YF/JE SA14-14-2 (E-138 K---> mutant).

Fig. 20 is a schematic representation of a two plasmid system for generating chimeric YF/DEN-2 virus. The strategy is essentially as described for the YF/JE chimeric virus.

Fig. 21 is a schematic representation of the structure of modified YF clones designed to delete portions of the NS1 protein and/or express foreign proteins under control of an internal ribosome entry site (IRES). The figure shows only the E/NS1 region of the viral genome. A translational stop codon is introduced at the carboxyl terminus of the envelope (E) protein. Downstream translation is initiated within an intergenic open reading frame (ORF) by IRES-1, driving expression of foreign proteins (*e.g.*, HCV proteins E1 and/or E2). The second IRES (IRES-2) controls translational initiation of the YF nonstructural region, in which nested, truncated NS1 proteins (*e.g.*, NS1del-1, NS1del-2, or NS1del-3) are expressed. The size of the NS1 deletion is inversely proportional to that of the ORF linked to IRES-1.

Fig. 22 is a graph showing the neurovirulence phenotype of ChimeriVax-Den2 in outbred (CD-1) suckling mice inoculated by the I.C. route with 10,000 PFU/0.02 ml.

shadowed and black boxes, respectively. The chimeric YF/DEN3 genome was reconstituted by *in vitro* ligation of three fragments: the large BstBI-AatII portion of 5'3'/Den3/DXho plasmid, a PCR fragment containing the DEN3-specific part of 5.2/Den3 without the one nucleotide deletion (D1) digested with BstBI and EheI (an isoschizomer of NarI), and the large EheI-AatII fragment of YFM5.2 JE SA14-14-2. Ligation products were linearized with XhoI and then transcribed *in vitro* with SP6 RNA polymerase. Vero PM cells were transfected with *in vitro* RNA transcripts to recover the chimeric virus.

Fig. 31 is a schematic representation of an overview of construction of a YF/DEN4 chimera of the invention.

Fig. 32 is a schematic representation of a plasmid and fragment map relating to construction of a YF/DEN4 chimera of the invention.

Detailed Description

The invention provides chimeric flaviviruses that can be used in vaccination methods against flavivirus infection. Construction and analysis of chimeric flaviviruses of the invention, such as chimeras of yellow fever virus and Japanese Encephalitis (JE), Dengue types 1-4 (DEN 1-4), Murray Valley Encephalitis (MVE), St. Louis Encephalitis (SLE), West Nile (WN), Tick-borne Encephalitis (TBE), and Hepatitis C (HCV) viruses are described as follows.

Yellow fever (YF) virus is a member of the *Flaviviridae* family of small enveloped positive-strand RNA viruses. Flavivirus proteins are produced by translation of a single long open reading frame to generate a polyprotein, and a complex series of post-translational proteolytic cleavages of the polyprotein by a combination of host and viral proteases, to generate mature viral proteins (Amberg *et al.*, J. Virol. 73:8083-8094,

terminus of prM in the chimeras described below is central to the present invention. In particular, in the chimeras of the present invention, the length of the so-called "prM signal," which separates the two cleavage sites by 20 amino acids in YF (Figs. 1A and 1B), is substantially maintained, to ensure polyprotein proteolytic processing and subsequent growth of chimeric viruses that are created in a YF backbone. A hydrophobic domain within this signal serves to direct the translocation of prM into the ER lumen, where efficient signalase cleavage occurs only after cleavage at the NS2B-NS3 site in the capsid protein (Amberg *et al.*, J. Virol. 73:8083-8094, 1999; Figs. 1A and 1B).

In the chimeras of the present invention, only the regions encoding the membrane and envelope proteins (*i.e.*, the prME region) of a non-yellow fever flavivirus are used to replace the corresponding genes in a yellow fever virus clone. The prM signal of the yellow fever virus backbone is maintained. Another method, described in a patent application by C.J. Lai, WO 93/06214, suggests a universal approach to constructing chimeric flaviviruses, involving cloning the prME region of a donor virus into the backbone of an acceptor virus, such that the prM signal sequence is contributed by the incoming prM protein gene. This approach was illustrated using dengue 4 virus as the backbone (acceptor) and tick-borne encephalitis as the donor prME gene. As is illustrated in Fig. 2, the approach described in WO 93/06214 suggests that variability in this cloning strategy, with other chimeric models using flaviviruses as backbone, will have no effect on proper processing of the resulting polyprotein. That is, that flavivirus prM signals are exchangeable when producing viable chimeric viruses. However, attempts to use this approach with YF as a backbone for the insertion of prME genes of dengue 2 virus to create a chimera in which dengue 2 sequences were

length and sequence of the YF prM signal in the chimeras of the invention. That is, preferably, the length of the prM signal is 20 amino acids. Less preferably, the length of the prM signal is 15, 16, 17, 18, 19, or more than 20 amino acids in length. Also, it is preferable that the amino acid sequence of the YF prM signal is maintained in the chimeras of the invention, although this sequence can be modified using, for example, conservative amino acid substitutions. Preferably, the sequence of the prM signal is 100%, less preferably, 90%, 80%, 70%, 60%, 50%, or 40% identical to the YF prM signal.

As an example of construction of a chimera of the invention, Fig. 6 illustrates a YF/JE chimera in which the YF NS2B-NS3 protease recognition site is maintained. Thus, the recognition site for cleavage of the cytosolic from membrane-associated portions of capsid is homologous for the YF NS2B-NS3 enzyme. At the C/pr-M junction, the portion of the signalase recognition site upstream of the cleavage site is that of the backbone, YF, and the portion downstream of the cleavage site is that of the insert, JE. At the E/NS1 junction, the portion of the signalase recognition site upstream of the cleavage site is similar to that of the insert, JE (four of five of the amino acids are identical to those of the JE sequence), and the portion downstream of the cleavage site is that of the backbone, YF. It is preferable to maintain this or a higher level of amino acid sequence identity to the viruses that form the chimera. Alternatively, at least 25, 50, or 75% sequence identity can be maintained in the three to five amino acid positions flanking the signalase and NS2B-NS3 protease recognition sites.

Also possible, though less preferable, is the use of any of numerous known signal sequences to link the C and pre-M or E and NS1 proteins of the chimeras (see, *e.g.*, von Heijne, Eur. J. Biochem. 133:17-21, 1983; von

ensuring stable expression of YF sequences and generation of RNA transcripts of high specific infectivity.

Our strategy for construction of chimeras involves replacement of YF sequences within the YF5'3'IV and YFM5.2 plasmids by the corresponding JE sequences from the start of the prM protein (nucleotide 478, amino acid 128) through the E/NS1 cleavage site (nucleotide 2,452, amino acid 817). In addition to cloning of JE cDNA, several steps were required to introduce or eliminate restriction sites in both the YF and JE sequences to permit *in vitro* ligation. The structure of the template for regenerating chimeric YF (C)/JE (prM-E) virus is shown in Fig. 7. A second chimera, encoding the entire JE structural region (C-prM-E) was engineered using a similar strategy. The second chimera was not able to generate RNA of high infectivity.

1.1 Molecular Cloning of the JE Virus Structural Region

Clones of authentic JE structural protein genes were generated from the JE SA14-14-2 strain (JE live, attenuated vaccine strain), because the biological properties and molecular characterization of this strain are well-documented (see, *e.g.*, Eckels *et al.*, Vaccine 6:513-518, 1988; JE SA14-14-2 virus is available from the Centers for Disease Control, Fort Collins, Colorado and the Yale Arbovirus Research Unit, Yale University, New Haven, Connecticut, which are World Health Organization-designated Reference Centers for Arboviruses in the United States). JE SA14-14-2 virus at passage level PDK-5 was obtained and passaged in LLC-MK_2 cells to obtain sufficient amounts of virus for cDNA cloning. The strategy used involved cloning the structural region in two pieces that overlap at an *Nhe*I site (JE nucleotide 1,125), which can then be used for *in vitro* ligation.

(approximately 100 plaque-forming units/250 nanograms of transcript). The JE sequence from nucleotides 1,108 to 2,471 was subcloned from several independent PCR-derived clones of pBluescript/JE into YFM5.2(*Nar*I) using the unique *Nsi*I and *Nar*I restriction sites. YF5'3'IV/JE clones containing the YF 5' untranslated region (nucleotides 1-118) adjacent to the JE prM-E region were derived by PCR amplification.

To derive sequences containing the junction of the YF capsid and JE prM, a negative sense chimeric primer spanning this region was used with a positive sense primer corresponding to YF5'3'IV nucleotides 6,625-6,639 to generate PCR fragments that were then used as negative sense PCR primers in conjunction with a positive sense primer complementary to the pBluescript vector sequence upstream of the *EcoR*I site, to amplify the JE sequence (encoded in reverse orientation in the pBluescript vector) from nucleotide 477 (N-terminus of the prM protein) through the *Nhe*I site at nucleotide 1,125. The resulting PCR fragments were inserted into the YF5'3'IV plasmid using the *Not*I and *EcoR*I restriction sites. This construct contains the SP6 promoter preceding the YF 5'-untranslated region, followed by the sequence: YF (C) JE (prM-E), and contains the *Nhe*I site (JE nucleotide 1,125) required for *in vitro* ligation.

1.3 Engineering YFM5.2 and YF5'3'IV to Contain Restriction Sites for in vitro Ligation

To use the *Nhe*I site within the JE envelope sequence as a 5' *in vitro* ligation site, a redundant *Nhe*I site in the YFM5.2 plasmid (nucleotide 5,459) was eliminated. This was accomplished by silent mutation of the YF sequence at nucleotide 5,461 (T→C; alanine, amino acid 1820). This site was incorporated into YFM5.2 by ligation of appropriate restriction

utilize the *Nhe*I site for *in vitro* ligation. The entire JE region in the Nakayama clone was sequenced to verify that the replaced cDNA was authentic (Table 1).

1.5 Generation of Full-Length cDNA Templates, RNA Transfection, and Recovery of Infectious Virus

Procedures for generating full-length cDNA templates are essentially as described in Rice *et al.* (The New Biologist 1:285-96, 1989; also see Fig. 7). In the case of chimeric templates, the plasmids YF5'3'IV/JE(prM-E) and YFM5.2/JE are digested with *Nhe*I/*BspE*I and *in vitro* ligation is performed using 300 nanograms of purified fragments in the presence of T4 DNA ligase. The ligation products are linearized with *Xho*I to allow run-off transcription. SP6 transcripts are synthesized using 50 nanograms of purified template, quantitated by incorporation of ^{3}H-UTP, and integrity of the RNA is verified by non-denaturing agarose gel electrophoresis. Yields range from 5 to 10 micrograms of RNA per reaction using this procedure, most of which is present as full-length transcripts. Transfection of RNA transcripts in the presence of cationic liposomes is carried out as described by Rice *et al.* (*supra*) for YF 17D. In initial experiments, LLC-MK$_2$ cells were used for transfection and quantitation of virus, since we have determined the permissiveness for replication and plaque formation of the parental strains of YF and JE. Table 2 illustrates typical results of transfection experiments using Lipofectin (GIBCO/BRL) as a transfection vehicle. Vero cell lines have also been used routinely for preparation of infectious virus stocks, characterization of labeled proteins, and neutralization tests.

Amplification products from Vero cells were sent to the FDA (CBER) for preparation of the RMS in diploid, Fetal Rhesus lung cells. Fetal rhesus lung cells were received from the ATCC as cultured cells and

positions 177 and 264 occurred during subsequent passage, and appear to be genetically unstable between two SA14-14-2 virus passages in PHK and PDK cells, showing that this mutation is less critical for attenuation.

The nucleotide sequence of the E protein coding region of the RMS was determined to assess potential sequence variability resulting from viral passage. Total RNA was isolated from RMS-infected Vero cells, reversed transcribed, and PCR amplified to obtain sequencing templates. Several primers specific for SA14-14-2 virus were used in individual sequencing reactions and standard protocols for cycle sequencing were performed.

Sequence data revealed two single nucleotide mutations in the RMS E protein, when compared to the published SA14-14-2 JE strain sequence data. The first mutation is silent, and maps to amino acid position 4 (CTT to CTG); the second is at amino acid position 243 (AAA to GAA) and introduces a change from lysine to glutamic acid. Both mutations identified are present in the sequence of the JE wild type strains Nakayama, SA14 (parent of SA14-14-2), and JaOArS982 (Sumiyoshi *et al.*, J. Infect. Dis. 171:1144-1151, 1995); thus, they are unlikely to contribute to virulence phenotype. We conclude that *in vitro* passage in FRhL cells to obtain the RMS did not introduce unwanted mutations in the E protein.

1.7 Structural and Biological Characterization of Chimeric YF/JE Viruses

The genomic structure of chimeric YF/JE viruses recovered from transfection experiments was verified by RT/PCR-based analysis of viral RNA harvested from infected cell monolayers. These experiments were performed to eliminate the possibility that virus stocks were contaminated during transfection procedures. For these experiments, first-pass virus was

JE-specific hyperimmune ascitic fluid (ATCC) and YF-specific purified IgG (monoclonal antibody 2E10). Significant differences in the 50% plaque reduction titer of these antisera were observed for the chimeras when compared to the control viruses in these experiments (Table 3). The YF/JE SA14-14-2 chimeric vaccine candidate, as well as the Nakayama chimera and SA14-14-2 viruses, were neutralized only by JE ascitic fluid, whereas YF 17D was neutralized in a specific fashion by YF ascites and the monoclonal antibody (Table 3). Thus, epitopes required for neutralization are expressed in the infectious chimeric YF/JE viruses, and are specific for the JE virus.

1.8 Growth Properties in Cell Culture

The growth capacity of the chimeras has been examined quantitatively in cell lines of both primate and mosquito origin. Fig. 8 illustrates the cumulative growth curves of the chimeras on LLC-MK_2 cells after low multiplicity infection (0.5 plaque-forming units/cell). In this experiment, YF5.2iv (cloned derivative) and JE SA14-14-2 (uncloned) viruses were used for comparison. Both chimeric viruses reached a maximal virus yield of approximately one log higher than either parental virus. In the case of the YF/JE SA_{14}-14-2 chimera, the peak of virus production occurred 12 hours later than the YF/JE Nakayama chimera (50 hours vs. 38 hours). The YF/JE Nakayama chimera exhibited considerably more cytopathic effects than the YF/JE SA14-14-2 chimera on this cell line.

A similar experiment was carried out in C6/36 cells after low multiplicity infection (0.5 plaque-forming units/cell). Fig. 8 also illustrates the growth kinetics of the viruses in this invertebrate cell line. Similar virus yields were obtained at all points used for virus harvest in

788; and Non-structural proteins: amino acids 789-3421); (nucleotide sequence of RMS; the coding sequence is from nucleotide 119 to nucleotide 10381)) with YF-Vax®, cells were grown to 90% confluency and infected with RMS or YF-Vax® at an MOI of 0.1 pfu. Since MRC-5 cells generally grow slowly, these cells were kept for 10 days post inoculation. Samples were frozen daily for 7-10 days and infectivity determined by plaque assay in Vero cells. YF-Vax® and the YF/JE chimera grew to modest titers in MRC-5 cells (Fig. 10). The peak titer was ~4.7 $\log_{10}$ pfu for YF-Vax® achieved on the second day and was slightly lower, 4.5 $\log_{10}$ pfu, for the RMS after 6 days.

1.10 Growth Curve of YF/JE SA14-14-2 in FRhL cells with and without IFN-inhibitors

Fetal rhesus lung cells were obtained from the ATCC and propagated as described for MRC-5 cells. Growth kinetics of the RMS were determined with and without interferon inhibitors.

Double-stranded RNA appears to be the molecular species most likely to induce interferon (IFN) in many virus infected cells. Induction of interferon apparently plays a significant role in the cellular defense against viral infection. To escape cellular destruction, many viruses have developed strategies to down-regulate induction of interferon-dependent activities. Sindbis virus and vesicular stomatitis virus have been shown to be potent IFN inducers. Using chick embryo cells, mouse L cells, and different viral inducers of IFN, it was shown that 2-aminopurine (2AP) and indomethacin (IM) efficiently and reversibly inhibit IFN action (Sekellick *et al.*, J. IFN Res. 5:651, 1985; Marcus *et al.*, J. Gen. Virol. 69:1637, 1988).

approximately one week post-inoculation. No mortality or illness was observed among mice receiving either the JE SA14-14-2 parent or the chimera. The inocula used for the experiments were titered at the time of injection and a subgroup of the surviving mice were tested for the presence of neutralizing antibodies to confirm that infection had taken place. Among those tested, titers against the JE SA14-14-2 virus were similar for animals receiving either this strain or the chimera.

The results of additional experiments investigating the neurovirulence of the YF/JE SA14-14-2 chimera in mice are illustrated in Table 4. In these experiments, all of the mice inoculated with YF5.2iv died within 7-8 days. In contrast, none of the mice inoculated with YF/JE SA14-14-2 died during two weeks of post-inoculation observation.

The results of experiments investigating the neuroinvasiveness and pathogenesis of YF/JE chimeras are illustrated in Table 5. In these experiments, the chimeric viruses were inoculated into 3 week old mice at doses varying between 10,000 and 1 million plaque-forming units via the intraperitoneal route. None of the mice inoculated with YF/JE Nakayama or YF/JE SA14-14-2 died during three weeks of post-inoculation observation, indicating that the virus was incapable of causing illness after peripheral inoculation. Mice inoculated with YF/JE SA14-14-2 developed neutralizing antibodies against JE virus (Fig. 13).

In additional experiments testing the neurovirulence phenotype and immunogenicity of the RMS, 4-week old ICR mice (n=5) were inoculated by the i.c. route with 0.03 ml of graded doses of the RMS or YF-Vax® (Table 6). Control mice received only diluent medium by this route. Mice were observed daily and mortality rates were calculated.

Mice inoculated with YF-Vax® started to die on day 7 (Fig. 14A). The $icLD_{50}$ of unpassaged YF-Vax®, calculated by the method of Reed

induce significant titers of neutralizing antibodies 3 or 8 weeks post immunization, but antibodies were elicited at lower doses.

Very low doses (1.4-2.4 $\log_{10}$ PFU) of YF 17D vaccine elicited an immune response in mice 8 weeks after inoculation (Table 7). This result may indicate delayed replication of the vaccine in mice receiving low virus inocula. In contrast, the YF/JE SA14-14-2 chimeric vaccine in this dose range was not immunogenic. It is likely that the chimeric vaccine is somewhat less infectious for mice than YF 17D. However, when inoculated at an infective dose, the chimera appears to elicits a higher immune response. This may be due to higher replication in, or altered tropism for, host tissues. Animals that received two doses of JE-Vax® did not mount a significant antibody response. Only one animal in the 1:30 dose group developed a neutralizing titer of 1:10 eight weeks after immunization. This might be due to the route (s.c.) and dilution (1:30) of the vaccine.

1.13 Protection of YF/JE SA14-14-2 RMS immunized mice against challenge with virulent JE

The YF/JE SA14-14-2 RMS and other viruses were evaluated for immunogenicity and protection in C57/BL6 mice in collaboration with Dr. Alan Barrett, Department of Pathology, University of Texas Medical Branch, Galveston. Experimental groups are shown in Table 8. Ten-fold dilutions (10^2-10^5) of each virus were inoculated by the s.c. route into groups of 8 mice. Mice were observed for 21 days, at which time surviving animals were bled from the retro-orbital sinus and serum frozen for neutralization tests. The 50% immunizing dose (ID_{50}) for each virus and GMT was determined (see below).

1.14 Serological response

Sera from mice in groups shown in Table 8 were tested 21 days after immunization for neutralizing antibodies. N tests were performed as follows. Six-well plates were seeded with Vero cells at a density of 10^6 cells/well in MEM alpha containing 10% FBS, 1% nonessential amino acids, buffered with sodium bicarbonate. One hundred µl of each test serum (inactivated at 60°C for 30 minutes) diluted two-fold was mixed with an equal volume of virus containing 200-300 PFU. The virus-serum mixtures were incubated at 4°C overnight and 100 µl added to each well after removal of growth medium. The plates were overlaid after 1 hour incubation at 37°C with 0.6% agarose containing 3% fetal calf serum, 1% L-glutamine, 1% HEPES, and 1% pen-strep-amphotericin mixed 1:1 with 2x M199. After 4 days of incubation at 37°C, 5% CO_2, a second overlay containing 3% Neutral red was added. After appearance of plaques, the monolayer was fixed with 1% formaldehyde and stained with crystal violet. The plaque reduction titer is determined as the highest dilution of serum inhibiting ≥ 50% of plaques compared with the diluent-virus control.

Results are shown in Table 10 and Fig. 16. NT antibody responses in mice immunized with the YF/JE SA14-14-2 chimera showed a dose response and good correlation with protection. At doses of 4-5 logs, the chimeric vaccine elicited higher N antibody responses against JE than either SA14-14-2 virus or wild-type Nakayama virus. Responses were superior to those elicited by YF-Vax® against YF 17D virus. No prozone effect was observed in animals receiving the chimera or infectious-clone derived YF 5.2iv; responses at the highest vaccine dose (5 logs) were higher than at the next lower dose (4 logs). In contrast, mice that received

4.7 $\log_{10}$) pfu should not have viremia greater than 165,000 pfu/ml (approximately 16,500 mLD_{50}). None of the monkeys in the experiments had viremia of more than 15,000 pfu/ml, despite receiving 6 $\log_{10}$ pfu of the RMS.

Neutralizing antibody titers were measured at 2 and 4 weeks post inoculation (Fig. 18). All monkeys seroconverted and had high titers of neutralizing antibodies against the inoculated viruses. The level of neutralizing antibodies in 2 of 3 monkeys in both groups exceeded a titer of 1:6,400 (the last dilution of sera tested) at 4 weeks post inoculation. The geometric mean antibody titers for ChimeriVax were 75 and 3,200 after 2 and 4 weeks respectively and were 66 and 4971 for the YF-Vax® for the same time points (Table 11).

Histopathological examination of coded specimens of brain and spinal cord were performed by an expert neuropathologist (Dr. I. Levenbook, previously CBER/FDA), according to the WHO biological standards for yellow fever vaccine. There were no unusual target areas for histopathological lesions in brains of monkeys inoculated with ChimeriVax™-JE. Mean lesion scores in discriminator areas were similar in monkeys inoculated with YF-Vax® (0.08) and monkeys inoculated with a 100-fold higher dose of ChimeriVaxTM-JE (0.07). Mean lesion scores in discriminator + target areas were higher in monkeys inoculated with YF-Vax® (0.39) than in monkeys inoculated with a 100-fold higher dose of ChimeriVax™-JE (0.11). These preliminary results show an acceptable neurovirulence profile and immunogenicity for ChimeriVax™-JE vaccine. A summary of the histopathology results is provided in Table 22.

1.18 Position 138 on the E protein

A single mutation of an acidic residue glutamic acid (E) to a basic residue, lysine (K) at position 138 on the E protein of JE virus results in attenuation (Sumyoshi *et al.*, J. Infect. Dis. 171:1144, 1995). Experiments were carried out to determine whether the amino acid at position 138 of the JE envelope protein (K in the vaccine chimera and E in the virulent Nakayama chimera) is a critical determinant for neurovirulence in mice. Chimeric YF/JE SA14-14-2 (K 138----> E) virus containing the single reversion of

K---->E at position 138 was generated from an engineered cDNA template. The presence of the substitution and the integrity of the entire E protein of the resulting virus was verified by RT/PCR sequencing of the recovered virus. A standard fixed-dose neurovirulence test of the virus was conducted in 4-week-old outbred mice by i.c. inoculation with 10^4 pfu of virus. The YF/JE SA14-14-2 and YF/JE Nakayama chimeric viruses were used as controls. The virulence phenotype of YF/JE SA14-14-2 (K-->E) was indistinguishable from that of its attenuated parent YF/JE SA14-14-2 in this assay, with no morbidity or mortality observed in the mice during the observation period (Fig. 19).

We conclude that the single mutation at position 138 to the residue found in the JE-Nakayama virus does not exert a dominant effect on the neurovirulence of the YF/JE SA14-14-2 chimera, and that one or more additional mutations are required to establish the virulent phenotype.

1.19 Other putative attenuation loci

Additional experiments to address the contributions of the other 6 residues (mentioned above) using the format described here were

a selective advantage by competing more effectively with the original vaccine virus and take over the culture. Therefore, mutant strains of the vaccine that grow better than the original vaccine may be selected by subculturing *in vitro*. One concern that addressed experimentally is whether such selective pressures might lead to mutant vaccine viruses with increased virulence.

In theory, molecular evolution should occur more rapidly for RNA viruses than DNA viruses because viral RNA polymerases have *higher* error rates than viral DNA polymerases. According to some measurements, RNA virus mutation rates approach one mutation per replication event. This is why an RNA virus can be thought of as a family of very closely related sequences (or "quasispecies"), instead of a single unchanging sequence (a "classical species").

Two different approaches can be taken to determine the sequence of an RNA virus:

1) purify viral genomic RNA from the culture supernatant, reverse-transcribe the RNA into cDNA and sequence this cDNA. This is the approach we have taken. It yields an averaged, or consensus sequence, such that only mutations which represent a large proportion (roughly, >20%) of the viruses in the culture can be detected.

2) Alternatively, cDNA can be cloned and individual clones sequenced. This approach would reveal the quasispecies nature of the vaccine by identifying individual mutations (deviations from the consensus sequence) in some proportion of the clones.

from P1 to P10, but at P18 it is back to the value seen at P8. One possible explanation for this observation is that a mutant bearing the H394R mutation gradually became as abundant as the original virus but was then out-competed by a new mutant bearing other mutations not present in the M or E genes and therefore, only detected as a rebound in the A/G ratio. We are reproducing these results by doing a second passaging experiment under identical conditions. It must also be noted that duplicate samples of viral genomic RNA were isolated, reverse-transcribed, amplified, and sequenced in parallel for each passage examined. Reported results were seen in both duplicate samples, arguing against any RT-PCR artifacts obscuring the data.

These observations show that minor genetic changes (one nucleotide substitution in the entire envelope E and M genes) have occurred in the JE sequences of the chimeric vaccine upon passaging, but that selective pressures did not lead to the loss of any of the attenuating mutations of the E gene.

1.24 Neurovirulence phenotype of passages 10 and 18

Groups of five female ICR mice, 3 to 4 weeks-old, received 30 µl i.c. of undiluted, P1, P10, or P18, as well as 30 µl of 10-fold dilutions. None of the mice injected with P1, P10, or P18 (doses ≥ 7 $\log_{10}$ pfu) showed any sign of illness over a five week period. As determined by back-titration, the doses administered (pfu) were measured as shown in Table 17.

Finally, the sequences of the entire genomes of the RMS and p18 were determined and found to be identical, except for the E-H394 mutation (Table 25). There are 6 nucleotide (NT) differences (NT positions are shaded) between the published YF 17D sequences and RMS shown in bold letters. Changes in positions 5461, 5641, 8212, and 8581 are silent and do not result in amino acid substitution, whereas changes in positions 4025 (ns2a) and 7319 (ns4b) result in amino acid substitutions from V to M and from E to K, respectively. Amino acid Methionine (M) at position 4025 is unique for RMS and is not found in any other YF strains, including parent Asibi virus and other yellow fever 17D strains (*e.g.*, 204, 213, and 17DD), whereas Lysine (K) at position 7319 is found in 17D204F, 17D213, and 17DD, but not in 17D204US or Asibi strain. Since the RMS is more attenuated than YF 17D with respect to neurovirulence, and thus has better biological attributes as a human vaccine, it is possible that the amino acid differences at positions 4025 and 7319 in the nonstructural genes of the yellow fever portion of the chimeric virus contribute to attenuation. Other workers have shown that the nonstructural genes of yellow fever virus play an important role in the attenuation of neurovirulence (Monath, "Yellow Fever," *in* Plotkin *et al.*, (Eds.), Vaccines, 2nd edition, W.B. Saunders, Philadelphia, 1998).

1.27 Experiment to Identify Possible Interference Between YF 17D and YF/JE SA14-14-2

It is well-established that yellow fever virus encodes antigenic determinants on the NS1 protein that induce non-neutralizing, complement-fixing antibodies. Passive immunization of mice with monoclonal anti-NS1 antibodies confers protection against challenge.

groups (n=8) were immunized with a single dose of YF-Vax® (0.1 ml of a 1:2 dilution of reconstituted vaccine, containing 4.4 $\log_{10}$ pfu, previously determined to induce the highest immune response to YF virus). Six groups (n=4) of mice (similar age, 3-4 weeks old) were kept as controls for booster doses at 3, 6, and 12 months post primary immunization.

All mice were bled 4 and 8 weeks after primary immunization and their neutralizing antibody titers were measured against homologous viruses in a plaque assay. 21/24 (87.5%) of the animals immunized with a single dose of ChimeriVax™-JE developed anti-JE neutralizing antibodies 1 month after immunization; at 2 months, 18/24 (75%) were seropositive. Geometric mean increased somewhat between 1 and 2 months post inoculation. In contrast, only 25%-33% of the mice immunized with YF-Vax® seroconverted and antibody responses were low. These results show that YF 17D virus and chimeric viruses derived from YF 17D are restricted in their ability to replicate in the murine host; however, when the envelope of JE virus is incorporated in the chimeric virus, the ability to replicate in and immunize mice is apparently enhanced. Mice receiving two doses of JE-Vax® developed high neutralizing titers against parent Nakayama virus, and titers increased between 1 and 2 months post immunization.

1.29 Secondary Immunization of ChimeriVax™JE and JE-Vax® Immunized Mice With YFVax®

Three months and six months after primary immunization with ChimeriVax-JE, mice were inoculated with YF-Vax® (1:2 dilution of a human dose containing 4.4 $\log_{10}$ pfu). Control mice not previously immunized and of identical age received ChimeriVax™JE only or

responded 6 months later to immunization with YF-Vax® and that the GMT and range of neutralizing antibody titers were similar to controls suggests that the chimeric vaccine imposed no significant barrier to yellow fever immunization.

2.0 Construction of cDNA Templates for Generation of Yellow Fever/Dengue (YF/DEN) Chimeric Viruses

Derivation of chimeric Yellow Fever/Dengue (YF/DEN) viruses is described as follows which, in principle, is carried out the same as construction of the YF/JE chimeras described above. Other flavivirus chimeras can be engineered with a similar strategy, using natural or engineered restriction sites and, for example, oligonucleotide primers as shown in Table 20.

2.1 Construction of YF/DEN Chimeric Virus

Although several molecular clones for dengue viruses have been developed, problems have commonly been encountered with stability of viral cDNA in plasmid systems, and with the efficiency of replication of the recovered virus. We chose to use a clone of DEN-2 developed by Dr. Peter Wright, Dept. of Microbiology, Monash University, Clayton, Australia, because this system is relatively efficient for regenerating virus and employs a two-plasmid system similar to our own methodology. (See Table 21 for a comparison of the sequences of Dengue-2 and YF/Den-2_{218} viruses; YF/Den-2_{218} contains the nucleotide and amino acid sequences of PUO-218. The NGC and PR-159 strains, which are also listed in Table 21, are other wild strains of dengue that differ from PUO-218 and can be used in the chimeras of the invention.) The complete sequence of this

sequence 5' untranslated and capsid sequence and a 3' *Tfi*I site, together with a 3' PCR fragment beginning with a *Tfi*I site at the amino terminus of the dengue-2 prM protein and the flanking dengue-2 prM protein sequence, were ligated into the YF5'3'IV plasmid after intermediate construction in pBluescript. Screening with *Tfi*I was used to confirm correct assembly of the chimeric junction in the final plasmid YF5'3'IV/DEN(prM-E).

2.2 Construction of Chimeric YF/DEN Viruses Containing Portions of Two DEN Envelope Proteins

Since neutralization epitopes against DEN viruses are present on all three domains of the E protein, it is possible to construct novel chimeric virus vaccines that include sequences from two or more different DEN serotypes. In this embodiment of the invention, the C/prM junction and gene encoding the carboxyl terminal domain (Domain III) of one DEN serotype (*e.g.*, DEN-2) and the N-terminal sequences encoding Domains I and II of another DEN serotype (*e.g.*, DEN-1) are inserted in the YF 17D cDNA backbone. The junctions at C/prM and E/NS1 proteins are retained, as previously specified, to ensure the infectivity of the double-chimera. The resulting infectious virus progeny contains antigenic regions of two DEN serotypes and elicits neutralizing antibodies against both.

2.3 Transfection and Production of Progeny Virus

Plasmid YF5'3'IV/DEN(prME) and YFM5.2/DEN(E'-E) were cut with SphI and AatII restriction enzymes, appropriate YF and dengue fragments were isolated and ligated *in vitro* using T4 DNA ligase. After

2.5 Growth Kinetics in Cell Culture

The growth kinetics of the YF/Den-2 chimera were compared in Vero and FeRhL cells (Fig. 16). Cells were grown to confluency in tissue culture flask (T-75). FeRhL cells were grown in MEM containing Earle's salt, L-Glu, non-essential amino acids, 10% FBS and buffered with sodium bicarbonate, and Vero cells were grown in MEM-Alpha, L-Glu, 10% FBS (both media purchased from Gibco/BRL). Cells were inoculated with YF/Den2 at 0.1 MOI. After 1 hour of incubation at 37°C, medium containing 3% FBS was added, and flasks were returned to a CO_2 incubator. Every 24 hours, aliquots of 0.5 ml were removed, FBS was added to a final concentration of 20%, and frozen for determination of titers in a plaque assay. Forty eight hours post infection CPE was observed in FeRhL cells and reached 100% by day 3. In Vero cells, CPE was less dramatic and did not reached 100% by the completion of the experiment (day 5). As shown, the YF/Den2 reached its maximum titer (7.4 $\log_{10}$ pfu/ml) by day 3 and lost about one log (6.4 $\log_{10}$ pfu/ml) upon further incubation at 37°C, apparently due to death of host cells and virus degradation at this temperature. The maximum virus titer in Vero cells was achieved by day 2 (7.2 $\log_{10}$ pfu/ml) and only half log virus (6.8 $\log_{10}$ pfu/ml) was lost on the following 3 days. This higher rate of viable viruses in Vero cells may be explained by incomplete CPE observed in these cells. In sum, the chimera grows well in approved cell substrate for human use.

Although mouse neurovirulence does not predict virulence/attenuation of dengue viruses for humans, it is important to determine the neurovirulence of a YF/Den-2 chimeric virus. YF 17D retains a degree of neurotropism for mice, and causes (generally subclinical) encephalitis in monkeys after IC inoculation. For vaccine development of a den/YF chimera it will be necessary to show that the construct does not exceed YF 17D in neuroinvasiveness and neurovirulence. Ultimately safety studies in monkeys will be required. In initial studies, we determined if insertion of the prME of the PUO218 into YF 17D vaccine strain will affect its neurovirulence for suckling mice (Table 24). Groups of 3, 5, 7, and 9 days old suckling mice were inoculated by the I.C. route with 10,000 pfu of YF/Den-2 or YF/JE SA14-14-2 chimera and observed for paralysis or death for 21 days. For controls similar age groups were inoculated either sham with medium (I.C. or I.P.) or with 1,000 pfu of unpassaged commercial YF vaccine (YF-Vax) by the I.P. route (it is not necessary to inoculate suckling mice with YF-Vax by the I.C. route because we have previously shown that this vaccine is virulent for 4-weeks old mice by this route).

As shown in Fig. 22, all suckling mice (3 to 7 days old) inoculated by the I.C. route with the YF/Den2 chimera died between 11 and 14 days post inoculation, whereas 8 out of 10 suckling mice (9 days old) survived. Similarly, all suckling mice (3-5 days old) inoculated with YF-Vax by the I.P. route, with a dose which was 10-fold lower than the YF/Den2 chimera, died between 11 to 13 days post inoculation (Fig. 23). All nine day old, as well as 8 out of 9 seven day old, mice inoculated with the YF-Vax survived. Similar results to the YF/Den2 chimera obtained with suckling mice inoculated with the YF/JE SA14-14-2 chimera.

viruses (produced by tissue culture passage or recombinant DNA technology). Although some of these candidates have shown promise in preclinical and human volunteers, development of a successful dengue vaccine remained to implemented.

Evaluating the immunogenicity and protective efficacy of the YF/Den2 chimera in monkeys should shed light on selection of appropriate prME genes (form wild type or attenuated strain) for construction of all 4 serotypes of chimeric dengue viruses.

2.7 Stability of prME genes of ChimeriVax™-D2 virus in vitro

The ChimeriVax™-D2 virus at passage 2 post transfection was used to inoculate a 25 cm^2 flask of Vero cells. Total RNA was isolated and the complete nucleotide sequence of the ChimeriVax™-D2 was determined (P3) and compared to the published sequence of the YF 17D virus (Rice *et al.*, Science 229:726-733, 1985). There was one nucleotide difference: at position 6898 there was an A in the chimera (P3), which was a C in the 17D nucleotide sequence. No difference in the prME region was found when the sequence of ChimeriVax™-D2 was compared to its parent dengue 2 virus (PUO218 strain). Also, no mutations were found in the prME genes of the chimera upon 18 passages in VeroPM cells. Within the YF genes, however, there was one silent mutation in position 6910 (C to A), and at position 3524 the P18 virus appeared to be heterozygous (both parent nucleotides, G and mutant A, were present). This would translate into a mixture of E and K amino acids at position 354 of the NS1 protein.

Similar to the passage 3 virus, the passage 18 virus was not neurovirulent for 4 week old outbred mice inoculated by the IC route (5

rhesus monkeys, which lasts between 3-6 days. Attenuation of dengue 2 viruses can therefore be estimated by comparing the level and duration of viremia with reference wild-type strains. These experiments clearly showed that core and non-structural proteins of YF 17D virus present in ChimeriVax™-D2 do not interfere with ChimeriVax™-D2 immunization.

2.9 Dose response effectiveness of ChimeriVax™-D2 in monkeys.

The goals of this experiment were to (i) determine the viremia profile of the vaccine candidate, using YF 17D and wild type dengue 2 virus controls, (ii) compare neutralizing antibody responses to the vaccine candidate and wildtype virus, and (iii) determine minimum dose required for protection against challenge with wild type dengue-2 virus. It was anticipated that these experiments would define the viremia profile of the ChimeriVax™-D2 virus in non-YF immune monkeys, and would determine whether immunization with a single dose results in protection of animals against challenge with a wild type dengue 2 virus. Protection in these experiments is defined as reduction of viremia in test monkeys compared to control viruses.

As is shown in table 28, all monkeys became viremic, and the duration of viremia was dose-dependent. The peak level of viremia for ChimeriVax™-D2 was between 1.3 to 1.6 $\log_{10}$ pfu, which was significantly lower than that of the wild type dengue virus (3.6 $\log_{10}$ pfu).

All monkeys developed anti-dengue 2 neutralizing antibodies by day 15. Lower dose of the vaccine resulted in lower GMTs, however, by day 30 post-immunization, all monkeys developed high titers of neutralizing antibodies, independent of the dose they received. Upon challenge, no viremia was detected in any immunized monkeys,

Individual mosquitoes were triturated in 1 ml of M199 media (Gibco BRL, Grand Island, New York) supplemented with 5% fetal calf serum, clarified by brief centrifugation, and then titrated in Vero cells to monitor virus replication.

Both JE SA14 and JE SA14-14-2 viruses replicated in *Cx. tritaeniorhynchus* following IT inoculation, reaching titers at day 14 of 6.7 and 6.0 $\log_{10}$ pfu/mosquito, respectively (Figure 24A). Additionally, IFA conducted on head squashes from JE SA14 and JE SA14-14-2-inoculated *Cx. tritaeniorhynchus* mosquitoes was positive for detection of JE virus antigen. In contrast, YF 17D and ChimeriVax™-JE did not replicate in *Cx. tritaeniorhynchus* mosquitoes. Virus titers declined rapidly following inoculation, and no virus was detectable by plaque titration assay in YF 17D or ChimeriVax™-JE-inoculated mosquitoes by days 1 and 2, respectively (Figure 24A). IFA analysis of head squashes from *Cx. tritaeniorhynchus* mosquitoes inoculated with ChimeriVax™-JE or YF 17D was negative for JE or YF virus antigens, supporting our observation that neither the chimera nor YF 17D replicate in this mosquito species.

ChimeriVax™-JE did replicate in IT-inoculated *Ae. albopictus* mosquitoes, reaching a titer of 5.2 $\log_{10}$ pfu/mosquito at day 18 (Figure 24B) and IFA results were weakly positive for both JE virus and YF virus antigens. The JE SA14 and JE SA14-14-2 viruses also replicated in *Ae. albopictus* mosquitoes, reaching maximum titers of 6.3 and 6.0 $\log_{10}$ pfu/mosquito, respectively. YF 17D virus did not replicate to high titers in *Ae. albopictus* mosquitoes, however, a low level of detectable virus was maintained (3.8 $\log_{10}$ pfu/mosquito at day 18) (Figure 24B) and IFA-stained head squashes were weakly positive for YF virus antigen. ChimeriVax™-JE and YF 17D inoculated IT into *Ae. aegypti* mosquitoes

Figures 25B and 25C illustrate growth of the viruses in orally exposed *Ae. albopictus* and *Ae. aegypti* mosquitoes, respectively. Only JE SA14 and JE SA14-14-2 viruses successfully infected and replicated in these species. For example, in *Ae. aegypti* mosquitoes on day 15, the titers of JE SA14 and JE SA14-14-2 viruses were 5.4 and 5.5 $\log_{10}$ pfu, respectively. In contrast, mosquitoes that had ingested 4.7 $\log_{10}$ pfu/mosquito of YF17D virus or 4.5 $\log_{10}$ pfu/mosquito of ChimeriVax™-JE virus failed to become infected.

In a separate experiment, *Ae. aegypti* and *Ae. albopictus* mosquitoes were orally exposed to JE SA14-14-2, YF 17D, and ChimeriVax™-JE viruses and processed after 22 days extrinsic incubation to permit growth to maximum virus titers. The results of this experiment are summarized in Table 30. Only JE SA14-14-2 virus was detectable in mosquitoes. Because ChimeriVax™-JE did not grow in any of the mosquito species tested, transmission studies were not performed.

Viruses recovered from *Ae. Albopictus* after IT or oral inoculation, or from *Ae. Aegypti* after IT inoculation, were identical to their parent ChimeriVax™-JE virus (Vero2FrhL1) in the prME region.

2.13 Amplification and sequencing of the "late replicating" ChimeriVax™-JE viruses isolated from mosquitoes

Ae. albopictus mosquitoes inoculated with ChimeriVax™-JE by IT or oral routes and *Ae. aegypti* inoculated with ChimeriVax™-JE by IT route, were harvested on day 15 post-inoculation. After triturating in 1 ml of M199 (supplemented with 5% fetal calf serum), samples were clarified by centrifugation, filtered through a 0.2 micron filter, and used to inoculate a T-25 cm^2 flask of VeroPM cells, passage 144 (0.5 ml/flask). After 1

moderate growth following IT inoculation into *Ae. aegypti* and *Ae. albopictus* mosquitoes, reaching titers of 3.6-5.0 $\log_{10}$ pfu/mosquito. There was no change in the virus genotype associated with replication in mosquitoes. Similar results were observed in mosquitoes of all three species that were IT inoculated or had orally ingested the YF 17D vaccine virus. In contrast, all mosquitoes either IT inoculated with, or orally fed, wild type and vaccine JE viruses became infected, reaching maximum titers of 5.4-7.3 $\log_{10}$ pfu/mosquito. The growth of ChimeriVax™-D2 in both *Ae. albopictus* and *Ae. aegypti* mosquitoes inoculated by IT or oral routes was also significantly lower than its parent wild type dengue 2 and YF17D vaccine viruses.

These results showed that ChimeriVax™-JE and ChimeriVax™-D2 viruses are restricted in their abilities to infect and replicate in these mosquito vectors. The low viremia caused by the viruses in primates and poor infectivity for mosquitoes are safeguards against secondary spread of the vaccine virus.

3.0 Construction of ChimeriVax™ YF/DEN-1

A yellow fever/dengue 1 (YF/DEN-1) chimeric virus was constructed using a novel technology, which differs from the approaches used to construct Yellow fever/Japanese encephalitis (YF/JE) chimeric viruses as described by Chambers *et al.* (J. Virol. 73:3095-4101, 1999; see above), and the construction of YF/DEN-4 chimera (see below). We used the same two plasmid system used to create YF/DEN-4. These plasmids first encoded the yellow fever (YF) genome as created by Rice *et al.* (New Biol. 1:285-296, 1989). Later, the structural membrane precursor and envelope protein genes, *i.e.*, the prME region, of the YF genome plasmids

full-length virus cDNA template for RNA transcription. All steps involving cDNA fragments, plasmids, and PCR products were carried out in a BL-2 lab designated for recombinant DNA work. Steps involving manipulations of infectious RNA and virus were carried out in a limited access BL-2+ virus lab.

3.1 Amplification of Dengue 1 sequence

Dengue 1 cDNA was synthesized from RNA using the Superscript II™ method. All primers for this experiment were synthesized by Life Technologies and are listed in Table 31. Upon arrival as lyophilized material, they were dissolved to 250 µM stock solutions using RODI-water. From this, 25 µM working solutions were made. The fragment encoding the SP6 promoter and the yellow fever capsid (Fragment A) was amplified using XL-PCR Reaction Kit TM (Perkin-Elmer Part#N808-0192), with 0.5 µl (250 ng) of pYF5'3'TV plus 3.5 µl RODI-water as template and primers 1 and 2 (see Table 31). The fragment encoding dengue 1 prM and 5' end of E (Fragment B) was amplified using the XL-PCR Reaction Kit™ (Perkin-Elmer Part#N808-0192) and primers 3 and 4. The fragment encoding the 3' end of the Dengue 1 envelope gene (Fragment F) was amplified using the same protocol, but with primers 5 and 7. The fragment encompassing the YF portion of pYFM5.2 (Fragment G) was amplified using the same protocol, but with primers 8 and 9 and 1 µl of pYFM5.2/2 with 39 µl water. The PCR for fragments F and G required an annealing temperature of 50°C and an extension time of 6.5 minutes. The PCR reaction was performed using the following master mixes for each reaction.

Fragment	Approximate Size (kb)
A	0.94
B	0.65
F	1.3
G	6.0

Forty µl of each fragment was then separated on a 1% Agarose/TAE gel and purified using the QIAquick Gel Extraction Kit (Qiagen cat#28704). Next, the concentrations of the purified fragments were determined by UV absorption using 1:40 dilutions in RODI-water.

Sample	A280	A260	280/260	260/280	Concentration
Fragment A	0.0116	0.0260	0.4453	2.2457	52 ng/µl
Fragment B	0.0076	0.0202	0.3782	2.6440	40.4 ng/µl
Fragment F	0.0160	0.0335	0.4785	2.0898	67 ng/µl
Fragment G	0.0199	0.0380	0.5242	1.9076	76 ng/µl

3.2 Recombinant PCR

To create a fusion between the yellow fever capsid and DEN-1 prM, a recombinant PCR technique known as overlap-extension PCR was used to create Fragment E. The same basic UM and LM were used, and primers 1 and 4 replaced earlier primers. The same approach was used to create a fusion between fragment F and G, resulting in fragment H. For this, primers 5 and 9 were used. The cDNA mixes were as follows:

	Fragment E	Fragment B control	Fragment A control
H_2O	37.82 µl	38.97 µl	38.85 µl
Fragment A	1.15 µl	0 µl	1.15 µl
Fragment B	1.03 µl	1.03 µl	0 µl
Volume	40 µl	40 µl	40 µl

The capsid-prME fusion was cloned into the yellow fever plasmid needed, and after digestion of the purified Fragment E, as well as pYF5'3'IV, with the appropriate enzymes. The digested plasmid resulted in two bands. Lower bands seen contain a fragment of Japanese encephalitis virus equivalent to Fragment E. All restriction enzymes, buffers, and 100x BSA were from New England Biolabs. All the digestions were incubated in a Perkin-Elmer 480 cycler set to hold at 37°C overnight.

Fragment E Digest

Fragment E (600 ng)	5.8 µl
NEB Buffer 4	4 µl
10x BSA	4 µl
H_2O	24.2 µl
Not I	1 µl
Nhe I	1 µl
Volume	40 µl

pYFMIV5'3' Digest

pYFMIV5'3' (1.02 µg)	2 µl
NEB Buffer 4	2 µl
10x BSA	2 µl
H_2O	12 µl
Not I	1 µl
Nhe I	1 µl
Volume	20 µl

3.4 Vector Dephosphorylation

Calf Intestinal Phosphatase (CIP) from New England Biolabs (cat#290S) was diluted 1:10 in 1x CIP Buffer. One µl of this dilution was

pYFM-5'3' Control Ligation

Fragment E (97.5 ng)	0 µl
pYFM5'3' (50 ng)	3.0 µl
H_2O	14 µl
10x T4 ligase buffer	2 µl
T4 DNA ligase	1 µl
Volume	20 µl

3.7 Transformations

Ligation reactions were individually transformed into *E. coli* strain MC1061 (recA-). Briefly, an aliquot of MC1061 was removed from storage at -80°C and allowed to thaw on ice for one to two minutes. 0.9 ml of cold 0.1 M $CaCl_2$ was added to the cells. One hundred µl of cells was aliquoted into three 12 ml culture tubes on ice. Ten µl of each ligation reaction was added to each culture tube, leaving the third tube as a no DNA control. Culture tubes were left on ice for 30 minutes. The tubes were heat shocked in a water bath at 42°C for 45 seconds, and then were put back on ice for 2 minutes. 0.9 ml SOC medium was added to each culture tube and incubated at 225 pm in a shaking incubator at 37°C for 1 hour. Each transformation mix was aliquoted into 1.5 ml microcentrifuge tubes. One hundred µl of each mix was spread onto LB/Agar-Amp (100 µg/ml) plates and labeled as "neat." Each tube was spun at 14,000 rpm in a microcentrifuge for 2-3 seconds to pellet the cells. The supernatant was poured into the waste container and the pellet resuspended in the residual broth by pipetting up and down. This material was plated (approximately 100 µl) onto LB/Agar-Amp (100 µg/ml) plates and labeled as 10x. All plates were inverted in a 37°C incubator overnight.

3.9 Glycerol Stocks

One hundred twenty ml of LB-Amp (100 µg/ml) was then inoculated from a patch pYD1-5'3'/2 and shaken at 225 rpm overnight at 37°C. Two x 1 ml of this culture was then spun at 14 Krpm for 2-3 seconds to pellet the cells. These were resuspended in LB-Glycerol (30%) and frozen at -80°C.

3.10 MIDI Plasmid Preparation

Qiagen Midi-Prep was performed on the remaining culture using the following modified protocol.

1. Spin 150 ml of each culture at 7 Krpm in GSA rotor for 10 minutes to pellet.
2. Decant Supernatant
3. Resuspend pellet in 4 ml P1 buffer; Transfer to 50 ml Falcon tube.
4. Rinse centrifuge bottle with 1 ml P1 buffer and transfer to the Falcon tube.
5. Add 5 ml P2 buffer; invert gently; incubate 5 minutes at room temperature or until lysed (no more than 12 minutes).
6. Add 5 ml P3 buffer; mix as above; incubate 10 minutes on ice.
7. Transfer supernatant to Qiagen Syringe Filter; Let sit for 10 minutes.
8. Equilibrate Q-100 tip with 4 ml QBT.
9. Gently push plunger to filter supernatant onto Q-100 tip.
10. Allow to drain by gravity.
11. Wash with 10 ml 2x QC.

Both reactions were incubated in a 37°C block overnight. Five µl of each digestion was run out on a 1.5% Agarose/TAE gel to check for complete digestion. The digestion was then incubated at 65°C for 20 minutes to inactivate the enzyme. 2.5 µl Bst BI (NEB) was added to each reaction and placed at 65°C overnight. The expected results of the digest are as follows:

pYD1-5'3'/2	Fragment H
5.6 kb	7.2 kb
0.14 kb	0.1 kb

The largest band from each reaction was gel excised and the UV concentration was determined (as previously described).

Sample	A280	A260	280/260	260/280	Concentration
pYD1-5.2 fragment	0.0089	0.0154	0.5777	1.7309	30.8 ng/µl
Fragment H	0.0020	0.0018	1.1333	0.8824	3.6 ng/µl

There was not enough fragment H for the ligation. Another 50 µl of fragment H was cleaned over a Qiagen Qiaquick column and digested with Aat II and Bst BI as described previously. The digested fragment was then gel excised as before and the UV concentration determined.

Sample	A280	A260	280/260	260/280	Concentration
Fragment H	0.0000	0.0021	0.0000	NA	4.2 ng/µl

3.15 Linear cDNA extraction (RNAse free phase)

1. Add H_2O to 100 µl total volume.
2. Add 1/10th volume 3 M Sodium Acetate
3. Add 100 µl Phenol/Chloroform/Isoamyl Alcohol and spin at 14 Krpm for 5 minutes in a microcentrifuge. Extract upper layer into RNAse-free 1.5 ml tube. Repeat once.
4. Add 100 µl RNAse-free Chloroform. Spin at 14 Krpm for 5 minutes in a microcentrifuge. Extract upper layer into RNAse-free 1.5 ml tube. Repeat once.
5. Add 200 µl 100% RNAse-free ethanol.
6. Place on dry-ice/ethanol bath for 10 minutes.
7. Spin at 14 Krpm for 20 minutes in a microcentrifuge.
8. Wash with 200 µl 70% ethanol (RNAse-free).
9. Repeat 70% ethanol wash two more times.
10. Dry in Speed-Vac for 8 minutes (or until no more ethanol is present).
11. Resuspend in 22 µl nuclease free water from the SP6 kit listed below.

3.16 SP6 transcription

The following reaction was setup using the SP6 transcriptase kit (Epicentre) and Rnasin (Promega) in an RNAse-free 1.5 ml tube using RNAse-free tips in a BL-2 hood. The reaction was then placed in a 40°C water bath for 1 hour.

Total RNA control

PBS	250 µl
Lipofectin	20 µl
YF/JE total RNA	10 µl
Volume	280 µl

Lipofectin control

PBS	260 µl
Lipofectin	20 µl
Volume	280 µl

1. Allow reactions to sit at room temperature for 10 minutes, and then remove Media from the six well plates.
2. Wash 3 times with PBS.
3. Remove last of PBS.
4. Overlay with each lipofectin reaction (add the YF/DEN-1 RNA to the 2 x 10^6 cells/well plate). Add 280 µl media to the remaining wells.
5. Rock for 10 minutes at room temperature.
6. Wash 2 times with media.
7. Add 2 ml of media to each well and place in the 37°C CO_2 incubator for 4 days or more.

3.18 Harvest of the first Vero-PM passage (P1)

The supernatant from YF/DEN-1 was harvested on day 6 by splitting the 2 ml of supernatant between two cryovials (each containing 1

3. Five hundred ml of media (same as used for transfection) was added to the monolayer.

4. One ml of media only was added to a control flask.

5. The flasks were placed in a 37°C CO_2 incubator and rocked every 15 minutes for 1 hour.

6. Meanwhile, the remaining YF/DEN-1(P2) was harvested into 4 cryovials containing 1 ml FBS and 1 cryovial containing 0.5 ml FBS and labeled as YF/DEN-1(P2). The cell monolayer was harvested with 3 ml Trizol into 1.5 ml tubes. All vials were placed at -80°C in a box labeled YF/DEN-1.

7. After infection (Step 5), 4 ml of media was added to each flask and were transferred to the incubator for 4 or more days.

Harvest of P3

The supernatant from YF/DEN-(P3) was harvested on day 5 by splitting the 5 ml of supernatant between five cryovials (each containing 1 ml FBS), which were labeled YF/DEN-1(P3). The cell monolayer was harvested with 3 ml Trizol into 1.5 ml tubes. All vials and tubes were then placed at -80°C.

3.20 Virus Identification

The RNA from P3 was extracted using Trizol methods according to the manufacturer's protocol, RT-PCR was performed followed by sequencing of the YF/DEN-1 prME region 5', 3' junctions, inclusive. The expected sequence of the prME region was confirmed.

strain of YF that includes the 5' and 3' UTRs, the C gene, and the nonstructural protein genes, NS1-5, (a prerequisite for safety).

To engineer a YF/DEN3 chimera containing the prM-E cassette from DEN3 in place of the prM-E cassette of YF we first wanted to use the two-plasmid approach that was successful in previous studies where 17D YF virus (Rice *et al.*, New Biol. 1:285-296, 1989) and the YF/JE chimera (Chambers *et al.*, J. Virol. 73:3095-3101, 1999) were recovered following *in vitro* transcription and transfection. The DEN3 (strain PaH881/88) prM-E region was RT-PCR amplified in two adjacent fragments (Fig. 29). To determine consensus sequence of this region of the parental virus, the RT-PCT fragments were directly sequenced in both directions. Since oligonucleotide primers used to synthesize these fragments were designed based on the published sequence of the H87 reference strain of DEN3 (Osatomi *et al.*, Virology 176:643-647, 1990), actual viral sequences in the primer areas (at the beginning of prM, nucleotides 437-459; at the junction between the two fragments, nucleotides 1079-1131; and at the end of E, nucleotides 2385-2413) could not be determined. A total of 83 nucleotides changes were found compared to H87 strain. The rate of nucleotides differences, 4.44%, was similar to that (4.5%) reported previously by Delenda *et al.* (J. Gen. Virol. 75:1569-1578, 1994) who sequenced roughly 80% of PaH881/88 E gene. Although the majority of nucleotides differences in the 80% E area coincided with those found by Delenda *et al.* (V. Deubel, personal communication) (53 changes coincided), there were 4 additional changes that were not found by Delenda *et al.* In addition, we did not observe 3 of the changes reported by these authors. The PaH881/88 virus (a starting material in our experiments) was isolated from a patient by single amplification in mosquito AP61 cells. We propagated this virus in C6/36,

by PCR (other sequenced clones contained more mutations). Therefore, the standard ChimeriVax procedure for preparation of infectious *in vitro* RNA transcripts that employs two fragment ligation prior to *in vitro* transcription was modified. According to the standard protocol, the large BstBI-AatII fragment from 5.2/Den3 would be ligated with the large BstBI-AatII fragment of 5'3'/Den3/DXho (see in Fig. 30). Instead, to correct the deletion, three-fragment ligation was done (Fig. 30). The DEN-3 part of 5.2/Den3 was PCR-amplified on the #26 clone template with high-fidelity LA Taq polymerase and digested with BstBI and EheI (isoschizomer of NarI). The opposite PCR primer was expected to correct the deletion. Second fragment, corresponding to the NarI-AatII part of 5.2/Den3, was derived by digestion of YFM5.2 JE SA14-14-2 with EheI and AatII. The two fragments were ligated with the large BstBI-AatII fragment of 5'3'/Den3/ΔXho. Ligation products were digested with XhoI and transcribed *in vitro* with SP6 RNA polymerase.

Vero PM cells (at passage 149) grown in 6 well plates were transfected with the *in vitro* RNA transcripts. A first indication that the expected YF/DEN3 chimera was present was the appearance of CPE characteristic of other chimeras created to date based on the YF backbone. It was first noticeable on day 5 post-transfection and became apparent (~10% of detached and rounded cells) on day 7 when virus-containing medium was harvested (P1). Subsequent P2 and P3 viruses were obtained by infecting fresh monolayers of Vero PM cells (at passages 150 and 151, respectively) with the P1 and P2 viruses (1 and 0.5 ml of the viruses were used for each infection, respectively) and harvesting the virus when apparent CPE (~10%) was observable (on days 3 and 4 for P2, and day 3 for P3).

overcoming technical difficulties that are often encountered during cloning of genetic material from many flaviviruses in *E. coli* (especially dengue viruses). A viable 17D YF/DEN3 chimeric virus was recovered which is yet another successful example of the usefulness of the approach developed by Chambers *et al.* (Chambers *et al.*, J. Virol. 73:3095-3101, 1999; see above), in which the prM-E cassette of a heterologous flavivirus is inserted into the YF backbone such that the hydrophobic signal for prM remains YF-specific.

The materials and methods used to make and characterize the YF/DEN3 chimera are described as follows.

4.1 Virus and cells

DEN3 strain PaH881/88 was isolated from a patient by single amplification in AP61 (mosquito) cells. C6/36 cells were maintained in MEM (Gibco, Cat.# 11095-072) supplemented with 10% FBS (HyClone, Cat.# SH30070103) and 1x non-essential amino acids (Sigma, Cat.# M7145) (OraVax ML-8 medium, Lot.# 108H2308) at 28°C under 5% CO_2. DEN3 was passaged two times by infecting monolayers of C6/36 at an unknown MOI and harvesting virus-containing growth media on day 7 post-infection (P1 and P2) and one time by infecting C6/36 cells with the P2 virus at an MOI of ~ 0.01 pfu/cell and harvesting the medium on day 6 (P3; pronounced virus-specific CPE was observed in P3).
Virus-containing media were mixed with an equal volume of FBS, aliquoted and stored at 70°C. Following transfection/infection, Vero PM cells were maintained in MEM (Gibco, Cat.# 11095-080, Lot.# 1017611) supplemented with 5% heat-deactivated FBS (OraVax Lot.# AGE6578)

(ORAVAX/VOLTEMP/GROUPS/LABTECH/KOSTIA/folder "KP sequencing data"/Exp.##). With each area of interest, both DNA strands were sequenced and analyzed. Oligonucleotide primers are listed in Table 32.

Primers were ordered from Custom Primers (Life Technologies/GibcoBRL). In the names of primers, numbers indicate approximate localization of oligos on the DEN3 genome and "+/-" indicates orientation of each primer, with the following exceptions: oligo 5 is colinear with a region of YFM5'3' series of plasmids upstream from the NotI cloning site; oligos 6 (opposite) and 7 (direct) are YF-specific; the former corresponds to the end of YF C gene; oligos 15 (direct) and 16 (opposite) were designed for amplification and sequencing of inserts in the YFM5.2 series of plasmids and correspond to regions of the plasmids located within ~ 60 nucleotides upstream and downstream from the corresponding inserts, respectively; oligo 8 (direct) was used to mutate the XhoI site at nucleotide 1052 of the recombinant YF/DEN3 genome (within 5'3'/Den3 plasmid); and oligo 17 is colinear with the SP6 promoter and a few of the 5' terminal nucleotides from YF.

4.3 DNA manipulations

Standard molecular biology techniques were in accordance with Maniatis *et al.*, Molecular Cloning: a Laboratory Manual, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1992. All restriction enzymes, except for EheI (Fermentas) and T4 DNA ligase, were from New England Biolabs.

the resulting 5'3'/Den3, which is a pBR322-based plasmid maintained in *E. coli* MC1061RecA- cells, was sequenced using oligos 1, 2, 9, 10, and 17, and a correct clone (#3) was selected, which does not have any mutations compared to the consensus sequence.

Sequencing revealed that the DEN3-specific portion of 5'3'/Den3 contains an additional XhoI site located in the beginning of E gene (nucleotides 1007-1012 in DEN3 genome). Another XhoI site used for linearization prior to *in vitro* transcription (see below) is located at the end of YF sequence in 5'3'/Den3. The additional site was destroyed by silent oligonucleotide-directed mutagenesis (LA PCR; DEN3-specific C at nucleotide 1009 was changed to G) using oligo 8, resulting in a plasmid 5'3'/Den3/DXho. The entire region of the plasmid replaced during mutagenesis was sequenced with oligos 1, 2, 9, 10, and 17 and a clone (#10) was selected that does not have any mutations, except for the desired C to G nucleotide change.

4.5 Construction of 5.2/Den3 plasmid

The 3' terminal part of DEN3 prM-E region was RT-PCR amplified (XL PCR) on the P3 virion RNA template using primers 3 and 4. It starts with BstBI site introduced at nucleotides 1101-1106 for in-frame ligation with 5'3'/Den3/DXho plasmid and ends with a NarI site introduced precisely at the 3' end of E gene (nucleotides 2408-2413) for in-frame ligation with YF NS1. The NarI site that leads to Q to G change of the penultimate amino acid residue in the DEN3 E was used previously to generate YF/JE chimera (Chambers *et al.*, J. Virol. 73:3095-3101, 1999; see above). An NheI cloning site was placed upstream from the BstBI site. The consensus sequence of this DEN3 region was determined by

AS2606C2). RNA transcripts were analyzed by electrophoresis of 2 µl aliquots in 1% agarose gel. Monolayers of Vero PM cells grown in 6 well tissue culture plates were transfected with RNA transcripts using Lipofectin reagent (Gibco, Cat.# 18292-011). Following transfections, cells were incubated as is described above, and virus-containing media were harvested on indicated days post-transfection, mixed with equal volume of FBS, aliquoted and stored at -70°C.

5.0 Construction of ChimeriVaxTM YF/DEN-4

The purpose was to generate yellow fever/dengue 4 (YF/DEN-4) chimeric virus as a dengue vaccine candidate (see Figs. 31 and 32). To attain this, we used a technology derived from the construction of Yellow fever/ Japanese encephalitis (YF/JE SA 14-14-2) chimeric virus (Chambers *et al.*, J. Virol. 73:3095-3101, 1999). It consists of a two plasmid system which originally encoded the yellow fever (YF) genome. These YF plasmids were created by Charlie Rice (Rice *et al.*, New Biol. 1:285-296, 1989). The structural membrane precursor and envelope protein genes, *i.e.*, the prME portion, of the YF genome plasmids with that of JE SA14-14-2 sequence and used the resulting plasmids to produce RNA *in vitro*, which was then transfected into cells to produce live YF/JE chimeric virus. The system seemed suitable to construct other flavivirus chimeras using YF as backbone and here we describe the use of dengue 4 as a start point. The dengue 4 strain, #1228 isolated in 1978 in Indonesia and passaged twice in Mosquitoes, was passed once in C6/36 and total RNA was isolated to synthesize cDNA for PCR of the prME region as needed for cloning. Here we describe in detail the procedures for construction of the YF/DEN-4 chimera. The dengue 4 prME region was

A) was amplified using the XL-PCR Reaction Kit TM (Perkin-Elmer Part# N808-0192), 0.5 ml (250 ng) of template pYF5'3'IV plus 3.5 ml RODI-water, and primers 1 and 2. The fragment encoding dengue 4 prM and the 5' end of E (Fragment B) was amplified using the XL-PCR Reaction Kit TM (Perkin-Elmer Part#N808-0192) and primers 3 and 4. The fragment encoding the 3' end of dengue 4 envelope (Fragment C) was amplified using the same protocol but using primers 5 and 6. Each PCR reaction was performed as indicated in master mixes (see section 3.1, above).

For each reaction, the lower mix (LM) was added to a Perkin-Elmer thin-walled 0.2 ml tube. Next, Ampliwax 100 (Perkin-Elmer) was added to the tube, which was then placed in a Perkin-Elmer 2400 Thermal Cycler and heated to 80°C for 5 minutes, and then cooled to 4°C. The cDNA and UM were then added to the top of the wax layer. The tube was then cycled in a Perkin-Elmer 2400 as follows: 94°C, 1 minute; repeat 30 x (94°C, 15 seconds; 53°C, 15 seconds; 68°C, 3 minutes), 72°C, 4 minutes; 4°C, hold. The expected sizes of the PCR fragments for cloning were as follows:

Fragment	Approximate Size (kb)
A	0.940
B	0.650
C	1.300

Forty µl of each fragment was then separated on a 1% Agarose/TAE gel and purified using the QIAquick Gel Extraction Kit (Qiagen cat#28704). Next, the concentration of the purified fragments was determined by UV absorption using 1:40 dilutions in RODI-water.

Forty µl of Fragment E was then separated on a 1% Agarose/TAE gel and purified using the QIAquick Gel Extraction Kit (Qiagen cat#28704). Next, the concentration of the purified fragment was determined by UV absorption using 1:40 dilutions in RODI-water.

Sample	A280	A260	280/260	260/280	Concentration
Fragment E	0.0049	0.0110	0.4489	2.2276	22 ng/µl

5.3 Cloning of Fragments C and E into Yellow Fever Vectors

The fragments were then cloned into the yellow fever two-plasmid system by digestion of the purified Fragments C and E as well as the plasmids pYF5'3'IV and pYFM5.2/2 with appropriate restriction enzymes as shown below. The digested plasmids resulted in two bands. The smaller bands contain a fragment of Japanese encephalitis corresponding to either Fragment C or Fragment E for the new dengue 4 constructs. All restriction enzymes, buffers, and 100x BSA were from New England Biolabs. All the digestions were carried in a Perkin-Elmer 480 cycler set to hold at 37°C overnight.

Fragment E digest

Fragment E (528 ng)	27 µl
NEB buffer 4	4 µl
10x BSA	4 µl
H_2O	3 µl
Not I	1 µl
Nhe I	1 µl
Volume	40 µl

5.4 Vector Dephosphorylation

Calf Intestinal Phosphatase (CIP) from New England Biolabs (cat#290S) was diluted 1:10 in 1x CIP Buffer. One µl of this dilution was then added to the pYFMIV5'3' digest. 0.62 µl of stock CIP was added directly to the pYF5.2 digest. Both were incubated for 1 hour at 37°C. Then, 0.8 µl 125 mM EDTA was added to the two tubes and placed at 75°C for 10 minutes to inactivate CIP

5.5 Gel Excision

The digested PCR fragments were separated on a 1.0% Agarose/TAE gel, while the digested plasmids were separated on a 0.8% Agarose/TAE gel. All were purified using the QIAquick Gel Extraction Kit (Qiagen cat#28704).

5.6 Ligations

The digested Fragment E and pYF5'3'IV were ligated using T4 DNA Ligase (New England Biolabs cat#202S) to create pYD4-5'3'. The digested Fragment C and pYFM5.2 were ligated to create pYD4-5.2. All ligation reactions were incubated in a Perkin-Elmer 480 cycler set to hold at 16°C overnight.

pYF5.2 Control Ligation

Fragment C	0 µl
pYFM5.2 (70 ng)	8.8 µl
H_2O	8.2 µl
10x T4 ligase buffer	2 µl
T4 DNA ligase	1 µl
Volume	20 µl

5.7 Transformations

All four ligation reactions were transformed into *E. coli* strain MC1061 (recA-). An aliquot of MC1061 (OraVax Notebook 661-4) was removed from storage at -80°C and allowed to thaw on ice for one to two minutes. 0.9 ml of cold 0.1 M $CaCl_2$ was added to the cells. One hundred µl of cells was aliquoted into five 12 ml culture tubes on ice. Ten µl of each ligation reaction was added to each culture tube, leaving the fifth tube as a negative (no DNA) control. Culture tubes were left on ice for 30 minutes. The tubes were heat shocked in a water bath at 42°C for 45 seconds. The tubes were put back on ice for 2 minutes. 0.9 ml SOC medium was added to each culture tube and incubated at 225 pm in a shaking incubator at 37°C for 1 hour. Each transformation mix was aliquoted into 1.5 ml microcentrifuge tubes. One hundred µl of each was spread onto LB/Agar-Amp (100 µg/ml) plates and labeled as "neat." Each tube was spun at 14 Krpm in a microcentrifuge for 2-3 seconds to pellet the cells. The supernatant was poured into the waste container and the pellet resuspended in the residual broth by pipetting up and down. This material was plated (approximately 100 µl) onto LB/Agar-Amp (100 µg/ml) plates and labeled as 10x. All plates were inverted in a 37°C incubator overnight.

5.9 Glycerol Stocks

Five ml of LB-Amp (100 µg/ml) was then inoculated from a patch pYD4-5'3'/2 or pYD4-5.2/1 and shaken at 225 rpm overnight at 37°C. One ml of this culture was then spun at 14 Krpm for 2-3 seconds to pellet the cells. This was then resuspended in LB-Glycerol (30%) and frozen at -80°C.

5.10 MIDI Plasmid Preparation

Fifty µl of each glycerol stock was added to 150 ml LB-Amp (100 µg/ml) in separate 4 L flasks and shaken at 225 rpm overnight at 37°C. Qiagen Midi-Prep (Qiagen) was performed using the following modified protocol.

1. Spin 150 ml of each culture at 7 Krpm in GSA rotor for 10 minutes to pellet.
2. Decant Supernatant.
3. Resuspend pellet in 4 ml P1 Buffer; transfer to 50 ml Falcon tube.
4. Rinse centrifuge bottle with 2 ml P1 buffer and transfer to the Falcon tube.
5. Add 6 ml P2 buffer; invert gently; 5 minutes at room temperature or until lysed (no more than 12 minutes).
6. Add 6 ml P3; mix as above; 10 minutes on ice.
7. Transfer supernatant to Qiagen Syringe Filter; let sit for 10 minutes.
8. Equilibrate Q-100 tip with 4 ml QBT.
9. Gently push plunger to filter supernatant onto Q-100 tip.
10. Allow to drain by gravity.

YD4-5.2/1 (AatII digest)

pYD4-5.2 (10 µg)	35.5 µl
Buffer 4 (NEB)	5 µl
AatII (NEB)	2 µl
H_2O	7.5 µl
Volume	50 µl

Both reactions were incubated in a 37°C block for 2 hours. Five µl of each digest was run out on a 1.5% Agarose/TAE gel to check for complete digestion. The pYD4-5'3'/2 digest did not cut completely so the reaction was cleaned over a Qiaprep spin column (Qiagen). The digest was repeated using this material and 3 µl of Aat II. In addition, 3 µl of Aat II was added to the existing pYD4-5.2/1 reaction. Both were incubated in a 37°C block, overnight. After confirmation of digest on another gel (as previously described), 2.5 µl Bst BI (NEB) was added to each reaction and placed at 65°C for 3 hours. The results of the digest were as follows.

PYD4-5'3'/2	PYD4-5.2/1
5.6 kb	7.2 kb
	2.0 kb
0.14 kb	0.4 kb

The largest band from each reaction was gel excised as and the UV concentration was determined (as previously described).

5.12 Ligation

The following ligation reaction was setup using high concentration T4 DNA ligase (NEB). The ligations were incubated at 16°C overnight.

6. Place on dry-ice/ethanol bath for 10 minutes.
7. Spin at 14 Krpm for 20 minutes in a microcentrifuge.
8. Wash with 200 µl 70% ethanol (RNAse-free).
9. Repeat 70% ethanol wash two more times.
10. Dry in Speed-Vac for 8 minutes (or until no more ethanol is present).
11. Resuspend in 22 µl nuclease free water from the SP6 kit listed below.

5.15 SP6 transcription

The following reaction was setup using the SP6 transcriptase kit (Epicentre) and Rnasin (Promega) in an RNAse-free 1.5 ml tube using RNAse-free tips in a BL-2 hood. The reaction was then placed in a 40°C water bath for 1 hour.

Capping NTP solution	6 µl
10x buffer	2 µl
20 mM Cap Analog	3 µl
100 mM DTT	2 µl
Linearized DNA	5 µl
Rnasin	0.5 µl
SP6 transcriptase	2 µl
Volume	20.5 µl

5.16 RNA Transfection

Two six well tissue culture plates were seeded with Vero-PM (p#153 OraVax notebook#743-7) cells at 7.4 x 10^5 cells/well in growth

5. Rock for 10 minutes at room temperature.

6. Wash 2 times with media (MEM, Sodium Pyruvate, NEAA, P/S, 5% FBS).

7. Add 2 ml of media to each well and place in the 37°C CO_2 incubator for 4 days or more.

5.17 Chimeric Virus Harvest

The supernatant from YF/DEN-4 was harvested on day 6 by splitting the 2 ml of supernatant between two cryovials (each containing 1 ml FBS), which were labeled YF/DEN-4 (P1). The cell monolayer was harvested with 1 ml Trizol into a 1.5 ml tube. All vials and tubes were then placed at -80°C.

5.18 Amplification of YF/DEN-4

Passage #2

1. Three T-25 flasks containing Vero-PM cells (p#149) were obtained from the Cell Culture Facility. A frozen aliquot of YF/DEN-4 (P1) was removed from the -80°C freezer, thawed, then placed on ice. The same was done for an aliquot of YF/JE (frozen stock from the P1 control transfection).

2. Media was removed from each T-25 flask.

3. Five hundred µl of YF/DEN-4(P1) was added to the first flask, 500 µl of media (MEalphaM, NEAA, Sodium Pyruvate, 5% FBS, P/S) was added to the second flask, and 500 µl of YF/JE(P1) was added to the third flask.

23 ml Agarose (0.6% in water) was heated at 42°C in a 50 ml Falcon tube (tube #2). At the end of the 1 hour incubation, tube #1 was added to tube #2 and mixed thoroughly.

6. One ml of this overlay was then added to the edge of each well.

7. The plate was then put in the 37°C CO_2 incubator for 4 days.

8. The 2° overlay was made by preheating 25 ml M199(2X), 1.5 ml FBS, 1.5 ml Neutral Red, and 0.5 ml PSA at 42°C in a 50 ml Falcon tube (tube #1). Additionally, 21.5 ml Agarose (0.6% in water) was heated at 42°C in a 50 ml Falcon tube (tube #2). Tube #1 was added to tube #2 and mixed thoroughly.

9. One ml of this overlay was added to the center of each well.

10. It was then placed in the 37°C CO_2 incubator

Titration of P2 results

Instead of titer determination, plaques were picked for purification to segregate a mixed population of large and small plaques observed. The RNA from P2 was extracted using Trizol methods according to the manufacturer's protocol, RT-PCR was performed followed by sequencing of the YF/DEN-4 prME region 5', 3' junctions, inclusive. The expected sequence of the prME was confirmed.

6.0 Construction of Chimeric Templates for Other Flaviviruses

Procedures for generating full-length cDNA templates encoding chimeric YF/MVE, YF/SLE, YF/WN, YF/TBE viruses are similar to those described above for the YF/DEN-2 system. Table 20 illustrates the features of the strategy for generating YF 17D-based chimeric viruses. The unique restriction sites used for *in vitro* ligation, and the chimeric

exceeding the normal size of the genome (approximately 10,000 nucleotides), the detection strategy described below can be used. In addition, deletion of NS1 may be advantageous in the chimeric YF/Flavivirus systems described above, because partial deletion of this protein may abrogate the immunity to YF associated with antibodies against NS1, and thus avoid problems with vector immunity if more than one chimeric vaccine was to be needed in a given recipient, or if a YF vaccine had been previously given or needed at a future point.

The strategy involves creating a series of in-frame deletions within the NS1 coding region of the YFM5.2 plasmid, in conjunction with engineering a translational termination codon at the end of E, and a series of two IRESs (internal ribosome entry sites). One IRES is immediately downstream of the termination codon and allows for expression of an open reading frame within the region between E and NS1. The second IRES initiates translation from truncated NS1 proteins, providing expression of the remainder of the YF nonstructural polyprotein. These derivatives are tested for recovery of infectious virus and the construct with the largest deletion is used for insertion of foreign sequences (*e.g.*, HCV proteins) in the first IRES. This particular construct can also serve as a basis for determining whether deletion of NS1 will affect vector-specific immunity in the context of YF/Flavivirus chimeric viruses expressing prM-E, as described above.

The insertion of nucleotides encoding E1, E2, and/or E1 plus E2 HCV proteins is limited by the size of the deletion tolerated in the NS1 protein. Because of this, truncated HCV proteins can be used to enhance stability within the modified YF clone. The HCV proteins are engineered with an N-terminal signal sequence immediately following the IRES and a

deposit date of January 6, 1998: Chimeric Yellow Fever 17D/Dengue Type 2 Virus (YF/DEN-2; ATCC accession number ATCC VR-2593) and Chimeric Yellow Fever 17D/Japanese Encephalitis SA14-14-2 Virus (YF/JE A1.3; ATCC accession number ATCC VR-2594).

Table 1

Sequence comparison of JE strains and YF/JE chimeras

Virus	E 107	E 138	E 176	E 177	E 227	E 243	E 244	E 264	E 279	E 315
JE SA14-14-2	F	K	V	T	S	K	G	H	M	V
YF/JE SA14-14-2	F	K	V	A	S	E	G	H	M	V
YF/JE Nakayama	L	E	I	T	P	E	E	Q	K	A
JE Nakayama	L	E	I	T	P	E	E	Q	K	A
JE SA14	L	E	I	T	S	E	G	Q	K	V

Table 2

Characterization of YF/JE chimeras

Clone	Yield (µg)	Infectivity plaques/100 ng LLC-MK2	PBS log titer VERO	RNAse log titer VERO	DNAse log titer VERO
YF5.21v	5.5	15	7.2	0	7
YF/JE-S	7.6	50	6.2	0	6.2
YF/JE-N	7	60	5	0	5.4

Table 5

Neuroinvasiveness of YF/JE Chimeras

3 week old male ICR mice

	log dose (intraperitoneal)	% mortality	
YF/JE Nakayama	4	0	(0/5)
YF/JE Nakayama	5	0	(0/4)
YF/JE Nakayama	6	0	(0/4)
YF/JE SA14-14-2	4	0	(0/5)
YF/JE SA14-14-2	5	0	(0/4)
YF/JE SA14-14-2	6	0	(0/4)

Table 6

Doses and routes of virus inoculation into groups of 4-week-old ICR mice

Group	YF/JE s.c. log_{10} pfu	YF/JE i.c. log_{10} pfu	YF-VAX s.c. log_{10} pfu	YF-Vax i.c. log_{10} pfu	Total # mice
1	5	4.5	4.7	4.2	20
2	4	4	4.4	3.9	20
3	3	3	3.4	3.4	20
4	2	2	2.4	2.4	20
5	1	1	1.4	1.4	20
6	JE-Vax (BIKEN) 1:30, day 0, 7, s.c.				5
7	JE-Vax (BIKEN) 1:300, day 0, 7, s.c.				5
8	control s.c. (medium +10% FBS)				5
9	control i.c. (medium +10% FBS)				5

Table 8

Immunogenicity and protection vs. challenge

Mice were immunized on Day 0 with live vaccines and on days 0, 7, and 20 with JE-Vax, bled on day 21 and challenged on day 28.

Virus	No./group	Dose (pfu)	Route	Total no. mice
1. YF/JE (SA14-14-2 RMS)*	8	10^2-10^5	sc	32
2. YF 17D (iv5.2) (Vero)	8	10^2-10^5	sc	32
3. YF 17D (PMC)	8	10^2-10^5	sc	32
4. JE Nakayama	8	10^2-10^5	sc	32
5. JE SA14-14-2 (BHKP1)**	8	10^2-10^5	sc	32
6. YF/JE (Nakayama)#	8	10^2-10^5	sc	32
7. JE-Vax Connaught lot EJN*151B	8	100 ul 1:300 dil. on Day 0, 7 and 100 ul 1:5 dil. on D 20	sc	8
8. None (challenged)	8	.....	ip	8
9. None (unchallenged)	8	----------	----------	8

* YF/JE SA14-14-2 vaccine candidate

** Chinese live vaccine, passed once in BHK cells

Chimeric YF/JE virus, with prM-E insert of wild-type JE Nakayama

Table 10

Geometric mean neutralizing antibody titers, C57/BL6 mice 21 days after immunization with a single SC inoculum of graded doses of live virus vaccines and 1 day after the third dose of inactivated JE-Vax.

Vaccine	Dose (log_{10}PFU)	Antibody titer (GMT ± SD) vs. JEV	YF 17D
YF/JE SA14-14-2	5	44.8 ± 25.2	
	4	26.5 ± 23.1	
	3	6.2 ± 4.9	
	2	1.1 ± 0.35	
	1	1 ± 0	
SA14-14-2(BHK1)	5	2.5±4.3	
	4	3.5 ± 20.5	
	3	4.7 ± 15.5	
	2	1 ± 0	
JE Nakayama	5	1.32 ± 1	
	4	4 ± 4.0	
	3	1.6 ± 1.8	
	2	1 ± 0	
YF/JE-Nakayama	5	10 ± 70*	
	4	102.5 ± 45.7	
	3	76.8 ± 63.9	
	2	19.8 ± 8.1	
JE-Vax® (mouse brain)	3 doses**	2.8 ± 6.5	
YF-Vax®	5		11± 9.6
	4		13.8 ± 19.1
	3		4.3 ± 11.7
	2		1 ± 0
YF5.2iv (17D infect. clone)	5		29.3 ± 47.1
	4		11 ± 15.2
	3		8 ± 19.4
	2		2.1 ± 3.2
Controls	0	1 ± 0	

Table 12 Immunization and protection: rhesus monkeys

Screening HI test for flavivirus antibodies: negative

Group	N	Virus	Dose, route (log_{10}PFU/0.5 ml)	JE Challenge Day 60
1	3	YF/JE SA14-14-2	4.3 SC	5.0 IC
2	3	YF/JE SA14-14-2	5.3 SC	5.0 IC
3	4	Saline/sham	- SC	5.0 IC

- Viremia days 1-7 after immunization and challenge
- Neutralization test days 0, 15, 30, 45, and 60 after immunization and days 15 and 30 after challenge
- Necropsy day 30 post challenge

Table 14 JE neutralizing antibody responses, rhesus monkeys immunized with ChimeriVax™ by the SC Route

50% PRNT titers, heat-inactivated serum, no added complement

Monkey	Dose	Day post-inoculation		
	log_{10}PFU	Baseline	15	30
R423	4.3	<10	160	2560
R073		<10	80	640
R364		<10	160	320
R756	5.3	<10	20	320
R174		<10	640	2560
R147		<10	160	2560

Table 16

List of chimeric YF/JE mutants (1 to 9) constructed to identify residues involved in attenuation of the ChimeriVax™. Mutated amino acids on the E-proteins are shown in bold letters.

			Mutant Viruses										
Positions	Nakayam	ChimeriVax™	1	2	3	4	5	6	7	8	9	10	11
107	**L**	F	**L**	F	F	**L**	**L**	**F**	**L**	F	**L**	F	**L**
138	**E**	K	K	**E**	K	K	**E**	**E**	**E**	**E**	**E**	**E**	**E**
176	**I**	V	V	V	**I**	**I**	**V**	**I**	**I**	V	V	**I**	**I**
177	**T**	A	A	A	**T**	**T**	A	**T**	**T**	A	A	**T**	**T**
227	**P**	S	S	S	S	S	S	S	S	**P**	**P**	**P**	**P**
264	**Q**	H	H	H	H	H	H	H	H	**Q**	**Q**	**Q**	**Q**
279	**K**	M	M	M	M	M	M	M	M	**K**	**K**	**K**	**K**

Table 17

Dose administered i.c. (pfu)

Group	P1	P10	P18
Neat	$\geq 6x10^4$	$1x10^6$	$2x10^7$
10^{-1}	$\geq 6x10^3$	$1x10^5$	$2x10^6$

Table 18

Dose administered s.c. (pfu)

Group	RMS	P10	P18
Neat	$2x10^5$	$2x10^7$	$3x10^7$
10^5	$1x10^5$	$5x10^5$	$5x10^4$
10^4	$1x10^4$	$5x10^4$	$5x10^3$

Table 20

Engineering of YF/Flavivirus chimeras

Virus[7]	Chimeric C/prM junction[1]	Chimeric E/NS1 junction[2]	5' ligation[3]	3' ligation[4]	Sites[5] eliminated or (created)
YF/WN	X-cactgggagagcttgaaggtc (SEQ ID NO:1)	aaagccagttgcagccgcggtttaa (SEQ ID NO:2)	*Aat*II	*Nsi*I	
YF/DEN-1	X-aaggtagactggtgggctccc (SEQ ID NO:3)	gatcctcagtaccaaccgcggtttaa (SEQ ID NO:4)	*Aat*II	*Sph*I	*Sph*I in DEN
YF/DEN-2	X-aaggtagattggtgtgcattg (SEQ ID NO:5)	aaccctcagtaccaccccgcggtttaa (SEQ ID NO:6)	*Aat*II	*Sph*I	
YF/DEN-3	X-aaggtgaattgaagtgctcta (SEQ ID NO:7)	acccccagcaccaccccgcggtttaa (SEQ ID NO:8)	*Aat*II	*Sph*I	*Xho*I in DEN (*Sph*I in DEN)
YF/DEN-4	X-aaaaggaacagttgttctcta (SEQ ID NO:9)	acccgaagtgtcaaccgcggtttaa (SEQ ID NO:10)	*Aat*II	*Nsi*I	
YF/SLE	X-aacgtgaatagttggatagtc (SEQ ID NO:11)	accgttggtcgcacccgcggtttaa (SEQ ID NO:12)	*Aat*II	*Sph*I	*Aat*II in SLE
YF/MVE	X-aatttcgaaaggtggaaggtc (SEQ ID NO:13)	gaccggtgtttacagccgcggtttaa (SEQ ID NO:14)	*Aat*II	*Age*I	(*Age*I in YF)
YF/TBE	X-tactgcgaacgacgttgccac (SEQ ID NO:15)	actgggaacctcacccgcggtttaa (SEQ ID NO:16)	*Aat*II	AgeI	(*Age*I in YF)

1,2: The column illustrates the oligonucleotide used to generate chimeric YF/Flavivirus primers corresponding to the C/prM or E/NS1 junction. (See text). X = carboxyl terminal coding sequence of the YF capsid. The underlined region corresponds to the targeted heterologous sequence immediately upstream of the *Nar*I site (antisense - ccgcgg). This site allows insertion of PCR products into the Yfm5.2 (*Nar*I) plasmid required for generating full-length cDNA templates. Other nucleotides are specific to the heterologous virus. Oligonucleotide primers are listed 5' to 3'.

3,4: The unique restriction sites used for creating restriction fragments that can be isolated and ligated *in vitro* to produce full-length chimeric cDNA templates are listed. Because some sequences do not contain convenient sites, engineering of appropriate sites is required in some cases (footnote 5).

5: In parentheses are the restriction enzyme sites that must be created either in the YF backbone or the heterologous virus to allow efficient *in vitro* ligation. Sites not in parentheses must be eliminated. All such modifications are done by silent mutagenesis of the cDNA for the respective clone. Blank spaces indicate that no modification of the cDNA clones is required.

Table 22

Summary of histopathology results, monkeys inoculated with YF-Vax or YF/JE SA14-14-2 by the IC route

YF-Vax			ChimeriVax-JE		
Monkey No.	Discriminator area score	Discriminator plus target area score	Monkey No.	Discriminator area score	Discriminator plus target area score
N030	0.21	0.64	N191	0	0.17
N492	0.04	0.36	N290	0.09	0.06
N479	0	0.17	N431	0.13	0.09
Group means	0.08	0.39		0.07	0.11

Table 25 Summary of differences between virulent (Asibi) and attenuated (17D, 17DD, RMS, P18) yellow fever viruses

Gene	NT	Asibi	17D204US	RMS	P18	17D204F	17D213	17DD	AA
C	304	G	A	A	A	A	A	A	
	333	T	C	C	C	C	C	C	
non-M	469	A	A	.	.	A	A	G	
M	854	C	T	.	.	T	T	T	LF
	893	A	G	.	.	G	G	A	
E	1127	G	A	.	.	A	A	A	GR
	1140	C	T	.	.	T	T	C	AV
	1431	A	A	.	.	A	C	A	NT
	1436	G	G	.	.	G	G	A	DS
	1437	A	A			A	A	G	
	1453	C	T	.	.	T	T	T	AV
	1491	C	T	.	.	T	T	T	TI
	1538	C	C	.	.	C	C	A	
	1572	A	C	.	.	C	C	C	KT
	1750	C	T	.	.	T	T	T	
	1819	C	T	.	.	T	T	T	
	1870	G	A	.	.	A	A	A	MI
	1887	C	T	.	.	T	T	T	SF
	1946	C	T	.	.	T	T	C	PS
	1965	A	G	.	.	G	G	G	KR
	2110	G	G	.	.	G	G	A	
	2112	C	G	.	.	G	G	G	TR
	2142	C	A	.	.	A	A	A	PH
	2219	G	A	.	.	A	A	G	AT
	2220	C	C	.	.	C	C	T	TI
	2356	C	T	.	.	T	T	T	
NS1	2687	C	T	T	T	T	T	T	FL
	2706	A	G	G	G	G	G	G	
	3274	G	A	A	A	A	A	A	
	3371	A	G	G	G	G	G	G	VI
	3599	T	T	T	T	T	T	C	
	3613	G	A	A	A	A	A	A	
	3637	C	C	C	C	C	C	T	
ns2a	3817	G.A	G	G	G	G	G	G	
	3860	A	G	G	G	G	G	G	VM
	3915	T.A	T	T	T	T	T	T	
	4007	A	G	G	G	G	G	G	AT
	4013	C	T	T	T	T	T	C	FL
	4022	A	G	G	G	G	G	G	AT
	4025	G	G	A	A	G	G	G	VM
	4054	C	T	T	T	T	T	C	
	4056	C	T	T	T	T	T	T	FS
ns2b	4204	C	C	C	C	C	C	T	
	4289	A	C	C	C	C	C	C	LI
	4387	A	G	G	G	G	G	G	
	4505	A	C	C	C	C	C	C	LI
	4507	T	C	C	C	C	C	C	
NS3	4612	T	C	C	C	C	C	T	
	4664	G.A	G	G	G	G	G	G	
	4673	T	G	G	G	G	G	T	
	4942	A	A	A	A	A	A	G	
	4957	C	C	C	C	C	C	T	
	4973	G	G	G	G	G	G	A	
	5115	A	A	A	A	A	A	G	GR
	5131	G.T	G	G	G	G	G	G	MMI
	5153	A	G	G	G	G	G	A	VI
	5194	T	C	C	C	C	C	C	
	5225	A	C	C	C	C	C	C	
	5362	C	C	C	C	C	T	A	
	5431	C	T	T	T	T	T	T	

Table 26. Immunogenicity of ChimeriVax™-D2 passed in Vero cells for mice

Passage level[a]	Dose (Log_{10} pfu)	GMT[b] SC	IC
P3	5	1 ± 0[c]	61 ± 47
	4	1 ± 0	7 ± 15
P5	5	1 ± 0	46 ± 16
	4	1 ± 0	9 ± 20
P10	5	1.8 ± 7.7	46 ± 53
	4	1 ± 0	7 ± 15
P18	5	1 ± 0	53 ± 17
	4	1 ± 0	2 ± 16

a: ChimeriVax™-Den2 virus was passaged in Vero $_{PM}$ cells (P141-147) at MOI of 0.1-0.5 and harvested 2-3 days PI.
B: Geometric Mean Titers measured as the last dilution of sera which resulted in 50% reduction in number of virus plaques.
C: Titers less than 1:10

Table 27. Immunization and challenge of yellow fever immune monkeys

1st Vaccine	2nd Vaccine	Seroconversion Den2	YF	Viremic after wt Den2 challenge
YF 17D	ChimeriVax-Den2	3/3	3 3	0/3
YF 17D	Dengue-2 wt	4/4	4 4	0/4
YF 17D	YF 17D	0/3	3 3	3/3
YF 17D	None	0/2	2 2	2/2
None	None	0/2	0 2	2/2

Table 31

Primers (restriction sites are underlined)

#1) YFM5'3'(4.56)+
(GTGAGCATTGAGAAAGCGCCACGCTTC)(SEQ ID NO:17)

#2) YF0.481-
(TCCACCCGTCATCAACAGCATTCCCAAAATTAG)(SEQ ID NO:18)

#3) 1DE 0.42+
(GAATGCTGTTGATGACGGGTGGATTTCATCTGACCACACGAGGG)
(SEQ ID NO:19)

#4) 1DE 1.095-
(NheI/BstBI)(GCCGCTAGCTTTTCGAAGGACGGCAGGGTTTGTGACT
TC)(SEQ ID NO:20)

#5) 1DE 1.102+
(BstBI)(GCCATGCATTTCGAAAACTGTGCATCGAAGCTAAAATAT
C)(SEQ ID NO:21)

#7) 1DE 2.409FUSE-
(GGCGCATCCTTGATCGGCGCCAACCATGACTCCTAGGTACAG)(SE
Q ID NO:22)

#8) YF NarI+
(GGCGCCGATCAAGGATGCGCCATC)(SEQ ID NO:23)

#9) YF 8.545-
(CCAAGAGGTCATGTACTCAG)(SEQ ID NO:24)

#10) SP6YFa+
(ATTTAGGTGACACTATAGAGTAAATCCTGTGTGCTAATT)(SEQ ID
NO:25)

5'-GAGTATTGTCCCATGCTG (SEQ ID NO:38)

14 KPsD3/ 2.1+

5'-GGAATTGGAGACAAAGCC (SEQ ID NO:39)

15 KPs5.2 0.23+

5'-TGGATAGTGGACAGACAGTGG (SEQ ID NO:40)

16 KPs5.2 1.66-

5'-CTCTAAATATGAAGATACCATC (SEQ ID NO:41)

17 SP6-yfa

5'-ATTTAGGTGACACTATAGAGTAAATCCTGTGTGCTAATT (SEQ ID NO:42)

Table 34

#1) YFM5'3'(4.56)+ (GTGAGCATTGAGAAAGCGCCACGCTTC)(SEQ ID NO:43)

#2) YF0.481- (TCCACCCGTCATCAACAGCATTCCCAAAATTAG)(SEQ ID NO:44)

#3) 4DE 0.432+ (GAATGCTGTTGATGACGGGTGAATTTCACCTGTCAACAAGAGACGG) (SEQ ID NO:45)

#4) 4DEE 1.095- (GCCGCTAGCGGTTCGAAATAGAGCCACTTCCTTGGCTGT)(SEQ ID NO:46)

#5) 4DE 1.102+ (GCCGCTAGCTTCGAACCTATTGCATTGAAGCCTCGATATC)(SEQ ID NO:47)

#6) 4DE 2.409- (GCCGCCGGCGCCAACTGTGAAACCTAGAAACACAG)(SEQ ID NO:48)

#7) sp6YFa+(ATTTAGGTGACACTATAGAGTAAATCCTGTGTGCTAATT)(SEQ ID NO:49)

(Gresikova *et al.*, "Tick-borne Encephalitis," In *The Arboviruses, Ecology and Epidemiology*, Monath (ed.), CRC Press, Boca Raton, Florida, 1988, Volume IV, 177-203), the vaccine virus can be administered by a mucosal route to achieve a protective immune response. The vaccine can be administered as a primary prophylactic agent in adults or children at risk of flavivirus infection. The vaccines can also be used as secondary agents for treating flavivirus-infected patients by stimulating an immune response against the flavivirus.

It may be desirable to use the yellow fever vaccine vector system for immunizing a host against one virus (for example, Japanese Encephalitis virus) and to later reimmunize the same individual against a second or third virus using a different chimeric construct. A significant advantage of the chimeric yellow fever system is that the vector will not elicit strong immunity to itself. Nor will prior immunity to yellow fever virus preclude the use of the chimeric vaccine as a vector for heterologous gene expression. These advantages are due to the removal of the portion of the yellow fever vaccine E gene that encodes neutralizing (protective) antigens to yellow fever, and replacement with another, heterologous gene that does not provide cross-protection against yellow fever. Although YF 17D virus nonstructural proteins may play a role in protection, for example, by eliciting antibodies against NS1, which is involved in complement-dependent antibody mediated lysis of infected cells (Schlesinger *et al.*, J. Immunology 135:2805-2809, 1985), or by inducing cytotoxic T cell responses to NS3 or other proteins of the virus, it is unlikely that these responses will abrogate the ability of a live virus vaccine to stimulate neutralizing antibodies. This is supported by the facts that (1) individuals who have been previously infected with JE virus respond to vaccination with YF 17D similarly to persons without previous

therapy methods to introduce therapeutic gene products into a patient's cells and in cancer therapy. In these methods, genes encoding therapeutic gene products are inserted into the vectors, for example, in place of the gene encoding the prM-E protein.

Yellow fever 17D virus targets cells of the lymphoid and reticuloendothelial systems, including precursors in bone marrow, monocytes, macrophages, T cells, and B cells (Monath, "Pathobiology of the Flaviviruses," pp. 375-425, *in* Schlesinger & Schlesinger (Eds.), *The Togaviridae and Flaviviridae*, Plenum Press, New York 1986). The yellow fever 17D virus thus naturally targets cells involved in antigen presentation and immune stimulation. Replication of the virus in these cells, with high-level expression of heterologous genes, makes yellow fever 17D vaccine virus an ideal vector for gene therapy or immunotherapy against cancers of the lymphoreticular system and leukemias, for example. Additional advantages are that (1) the flavivirus genome does not integrate into host cell DNA, (2) yellow fever virus appears to persist in the host for prolonged periods, and (3) that heterologous genes can be inserted at the 3' end of the yellow fever vector, as described above in the strategy for producing a Hepatitis C vaccine. Yellow fever 17D virus can be used as a vector carrying tumor antigens for induction of immune responses for cancer immunotherapy. As a second application, yellow fever 17D can be used to target lymphoreticular tumors and express heterologous genes that have anti-tumor effects, including cytokines, such as TNF-alpha. As a third application, yellow fever 17D can be used to target heterologous genes to bone marrow to direct expression of bioactive molecules required to treat hematologic diseases, such as, for example, neutropenia; an example of a bioactive molecule that can be used in such an application is GM-CSF, but

1. A chimeric live, infectious, attenuated virus, comprising:

a yellow fever virus in which the nucleotide sequence encoding a prM-E protein is either deleted, truncated, or mutated so that functional yellow fever virus prM-E protein is not expressed, and

integrated into the genome of said yellow fever virus, a nucleotide sequence encoding a prM-E protein of a second, different flavivirus, so that said prM-E protein of said second flavivirus is expressed.

2. The chimeric virus of claim 1, wherein said second flavivirus is selected from the group consisting of a Japanese Encephalitis (JE) virus, a Dengue virus selected from the group consisting of Dengue types 1, 2, 3, and 4, a Murray Valley Encephalitis virus, a St. Louis Encephalitis virus, a West Nile virus, a Tick-borne Encephalitis virus (*i.e.*, a Central European Encephalitis virus or a Russian Spring-Summer Encephalitis virus), a Hepatitis C virus, a Kunjin virus, a Powassan virus, a Kyasanur Forest Disease virus, and an Omsk Hemorrhagic Fever virus.

3. The chimeric virus of claim 1, wherein said second flavivirus is a Dengue virus, and nucleotide sequences derived from said Dengue virus are derived from two or more different Dengue strains.

8. The use of claim 7, wherein said second flavivirus is selected from the group consisting of a Japanese Encephalitis (JE) virus, a Dengue virus selected from the group consisting of Dengue types 1, 2, 3, and 4, a Murray Valley Encephalitis virus, a St. Louis Encephalitis virus, a West Nile virus, a Tick-borne Encephalitis virus (*i.e.*, a Central European Encephalitis virus or a Russian Spring-Summer Encephalitis virus), a Hepatitis C virus, a Kunjin virus, a Powassan virus, a Kyasanur Forest Disease virus, and an Omsk Hemorrhagic Fever virus.

9. The use of claim 7, wherein second flavivirus is a Dengue virus, and nucleotide sequences derived from said Dengue virus are derived from two or more different Dengue strains.

10. The use of claim 7, wherein the nucleotide sequence encoding the prM-E protein of said second, different flavivirus replaces the nucleotide sequence encoding the prM-E protein of said yellow fever virus or comprises a mutation that prevents prM cleavage to produce M protein.

11. The use of claim 7, wherein the prM signal of said chimeric virus is that of yellow fever virus.

15. The nucleic acid molecule of claim 13, wherein second flavivirus is a Dengue virus, and nucleotide sequences derived from said Dengue virus are derived from two or more different Dengue strains.

16. The nucleic acid molecule of claim 13, wherein the nucleotide sequence encoding the prM-E protein of said second, different flavivirus replaces the nucleotide sequence encoding the prM-E protein of said yellow fever virus or comprises a mutation that prevents prM cleavage to produce M protein.

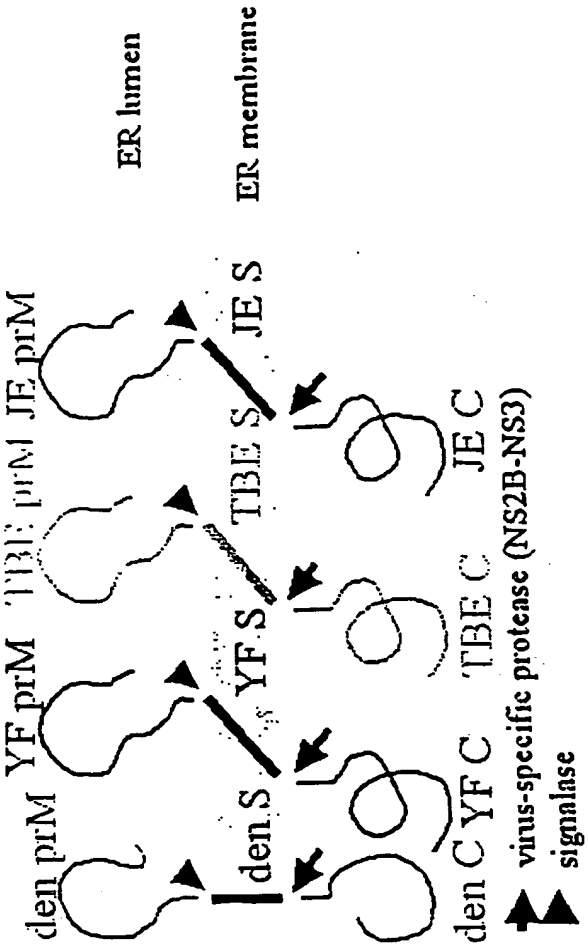
17. The nucleic acid molecule of claim 13, wherein the prM signal of said chimeric virus is that of yellow fever virus.

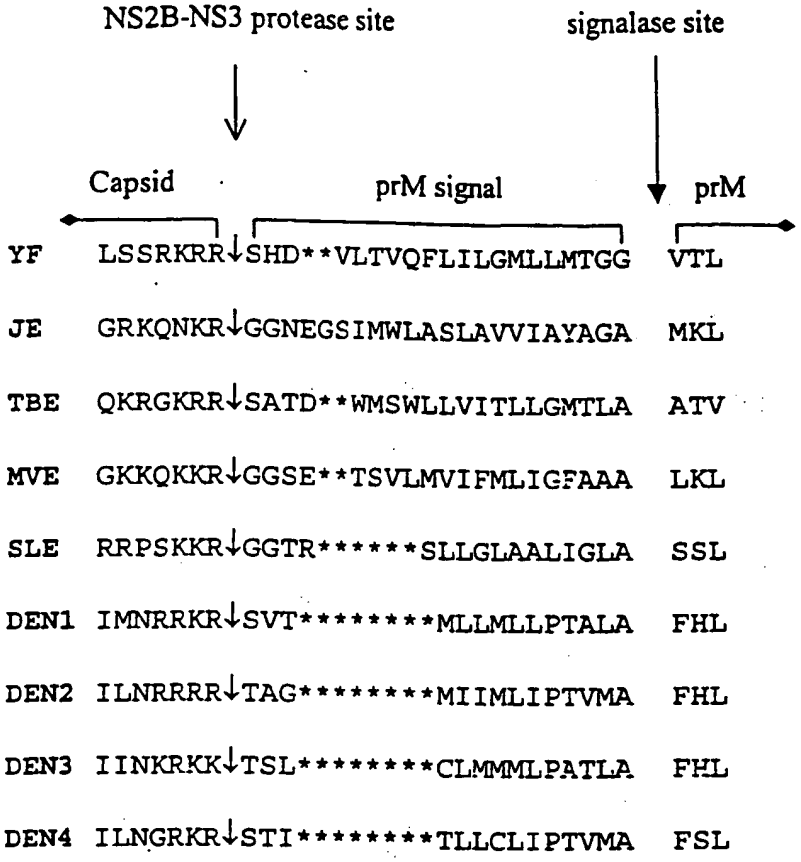
18. The nucleic acid molecule of claim 13, wherein NS2B-NS3 protease recognition site and the signal sequences and cleavage sites at the C/prM and E/NS1 junctions are maintained in construction of said chimeric flavivirus.

19. Use of a yellow fever virus vector comprising a gene encoding a gene product in the preparation of a medicament for producing said gene product in a cell of a patient.

20. The use of claim 19, wherein said cell is a cell of the lymphoid system or the reticuloendothelial system, or a precursor thereof.

Fig. 1A


Processing events at the C/prM junction of the parental viruses
ER lumen
ER membrane
den prM
YF prM
TBE prM
JE prM
den S
YF S
TBE S
JE S
den C
YF C
TBE C
JE C
virus-specific protease (NS2B-NS3)
signalase



Virus names abbreviations

YF = yellow fever
JE = Japanese encephalitis
TBE = Tick-borne encephalitis
MVE = Murray Valley encephalitis
SLE = Saint Louis encephalitis
DEN1-4 = dengue serotypes 1-4

Fig. 1B

Fig. 2

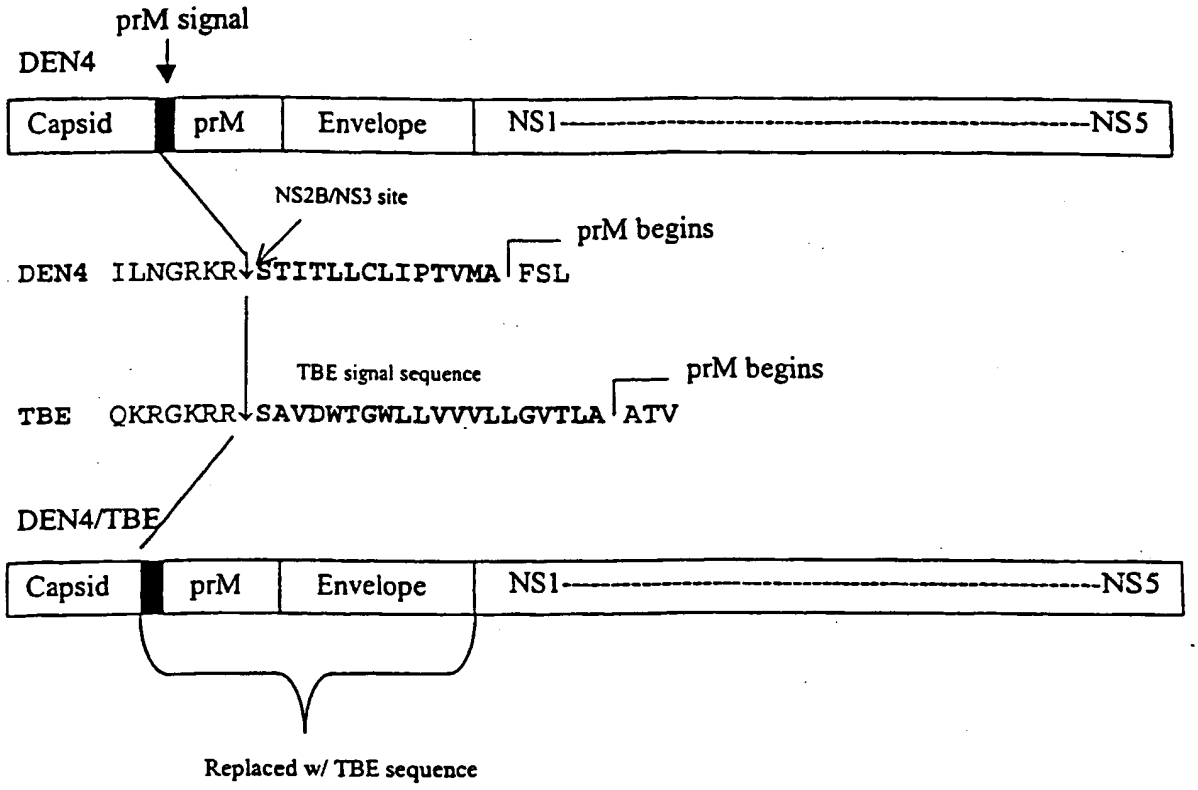

prM signal
DEN4
Capsid
prM
Envelope
NS1
NS5
NS2B/NS3 site
prM begins
DEN4 ILNGRKR↓STITLLCLIPTVMA FSL
TBE signal sequence
prM begins
TBE QKRGKRR↓SAVDWTGWLLVVVLLGVTLA ATV
DEN4/TBE
Capsid
prM
Envelope
NS1
NS5
Replaced w/ TBE sequence

Fig. 3

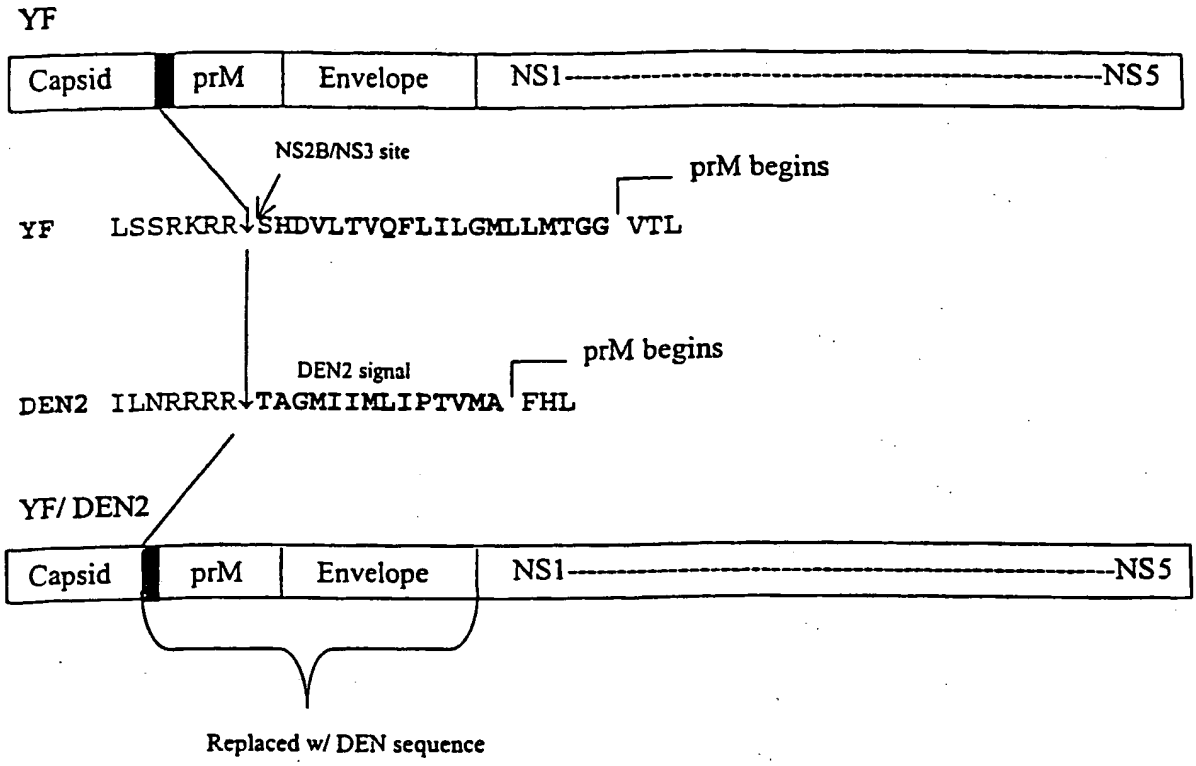

YF
Capsid
prM
Envelope
NS1
NS5
NS2B/NS3 site
prM begins
YF LSSRKRR↓SHDVLTVQFLILGMLLMTGG VTL
DEN2 signal
prM begins
DEN2 ILNRRRR↓TAGMIIMLIPTVMA FHL
YF/ DEN2
Capsid
prM
Envelope
NS1
NS5
Replaced w/ DEN sequence

Fig. 4

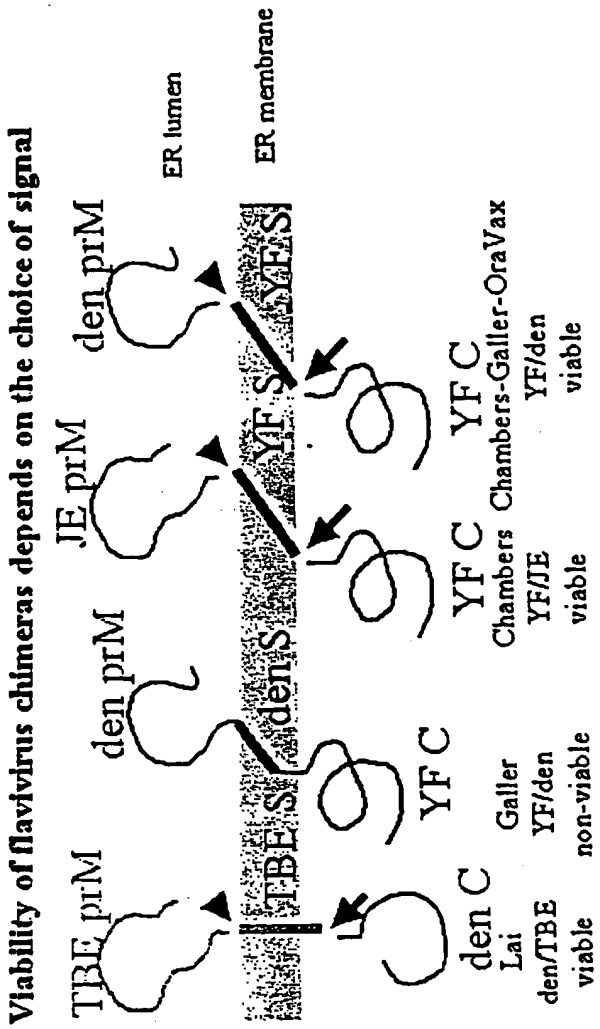
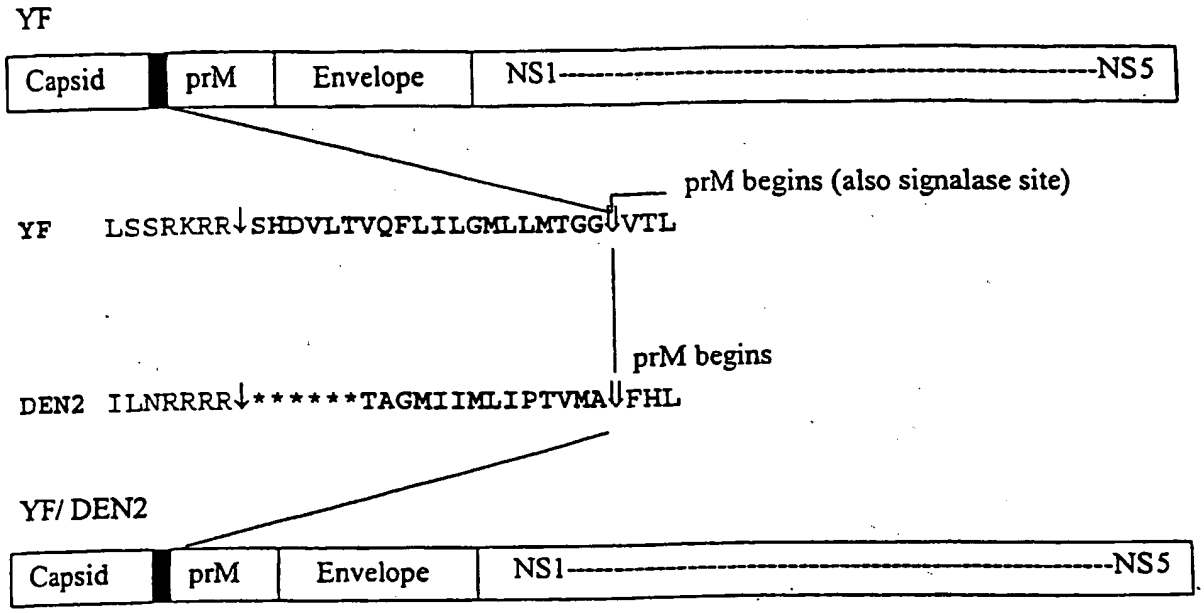
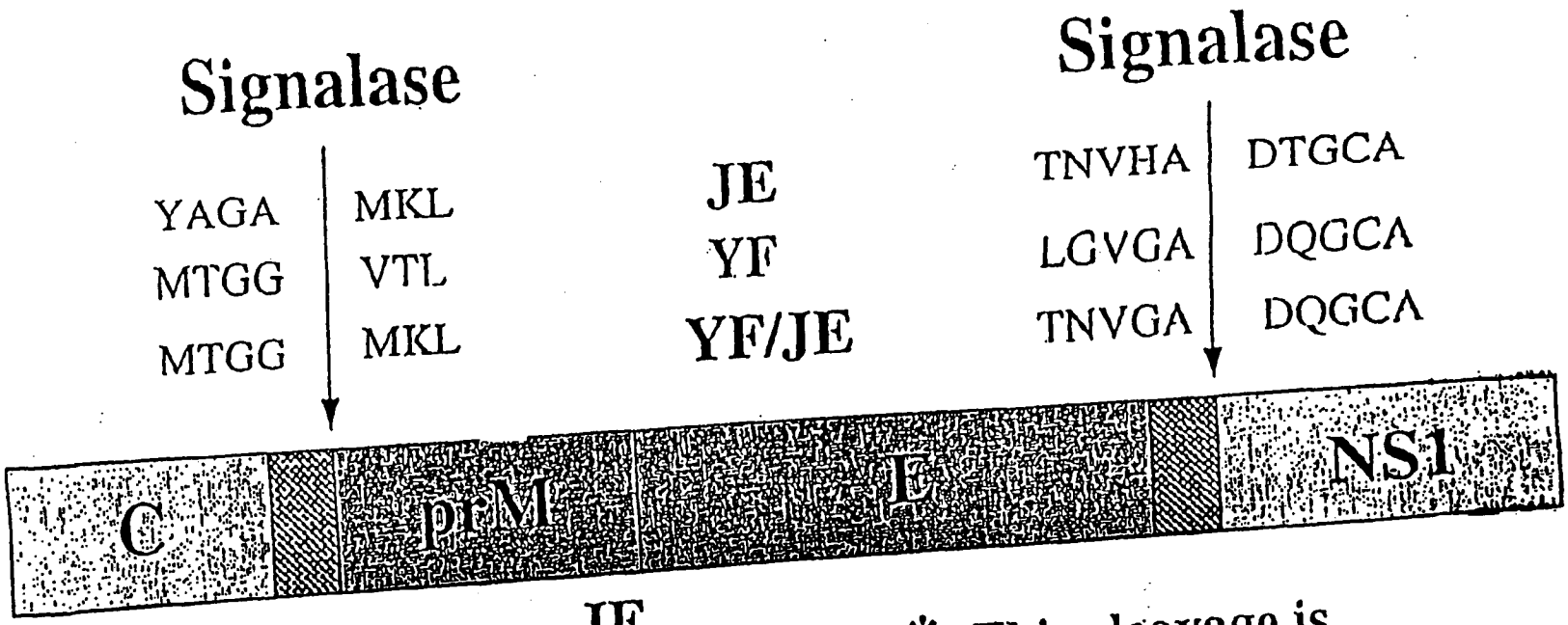

Viability of flavivirus chimeras depends on the choice of signal
TBE prM
den prM
JE prM
den prM
ER lumen
ER membrane
TBE S
den S
YF S
YF S
den C
YF C
YF C
YF C
Lai
den/TBE
viable
Galler
YF/den
non-viable
Chambers
YF/JE
viable
Chambers-Galler-OraVax
YF/den
viable

Fig. 5


YF
Capsid
prM
Envelope
NS1
NS5
prM begins (also signalase site)
YF LSSRKRR↓SHDVLTVQFLILGMLLMTGG⇓VTL
prM begins
DEN2 ILNRRRR↓******TAGMIIMLIPTVMA⇓FHL
YF/ DEN2
Capsid
prM
Envelope
NS1
NS5

Junction sequences of ChimeriVax™-JE (YF/JE) virus



*: This cleavage is prerequisite for efficient signalase-mediated processing at the C/prM junction

Fig. 6

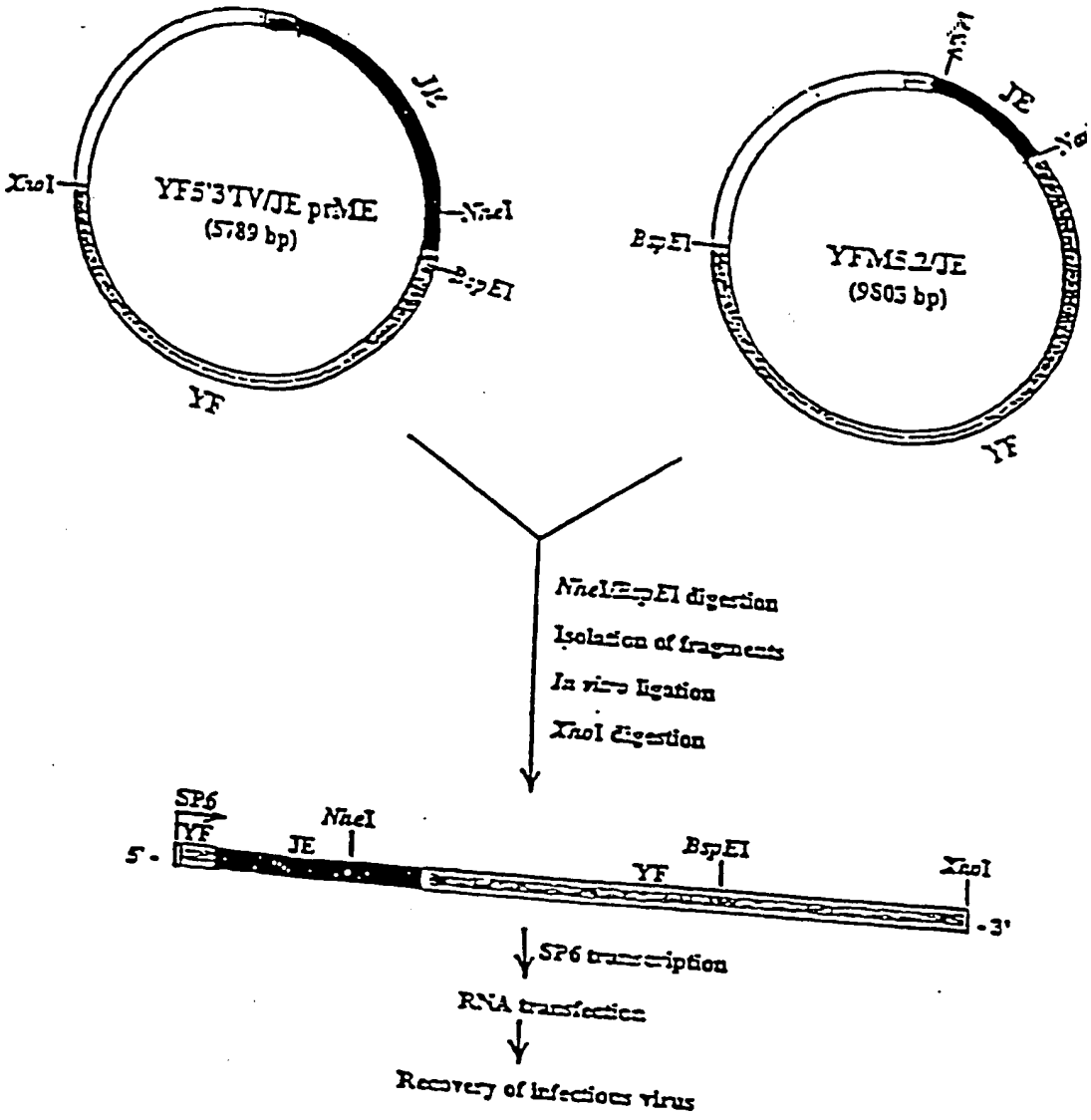
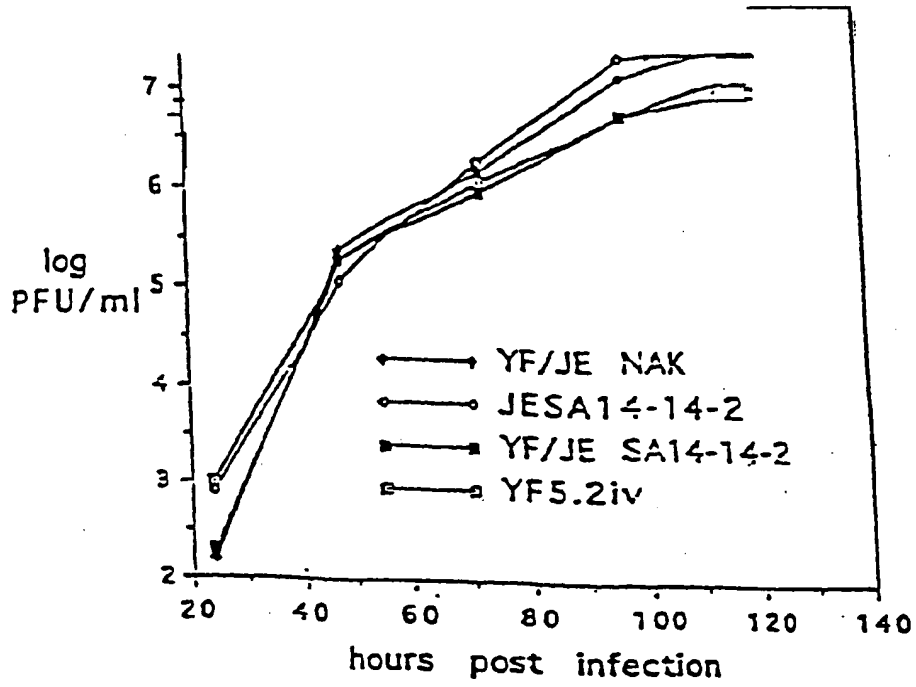

XhoI
YF5'3'TV/JE prM/E
(5789 bp)
NheI
BspEI
JE
YF
BspEI
YFM5.2/JE
(9503 bp)
JE
YF
NheI/BspEI digestion
Isolation of fragments
In vitro ligation
XhoI digestion
SP6
YF
5'
NheI
JE
BspEI
YF
XhoI
3'
SP6 transcription
RNA transfection
Recovery of infectious virus

Fig. 7


log
PFU/ml
7
6
5
4
3
2
20
40
60
80
100
120
140
hours post infection
YF/JE NAK
JESA14-14-2
YF/JE SA14-14-2
YF5.2iv

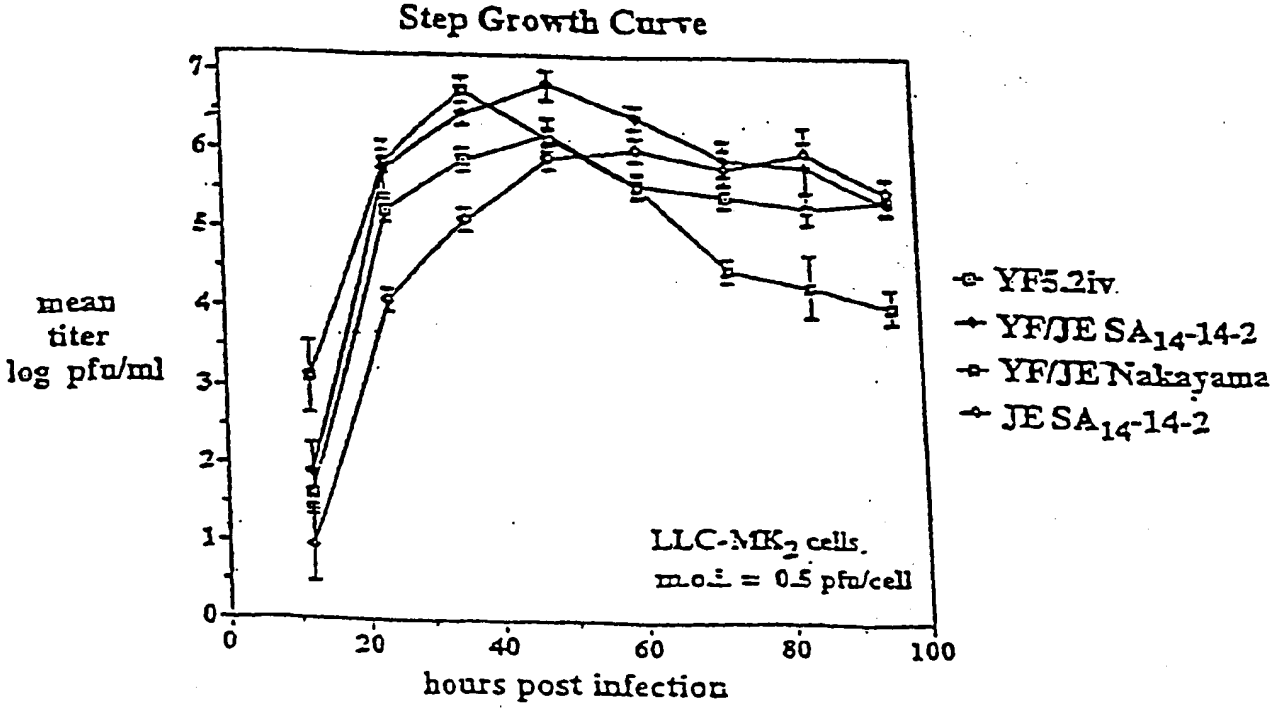
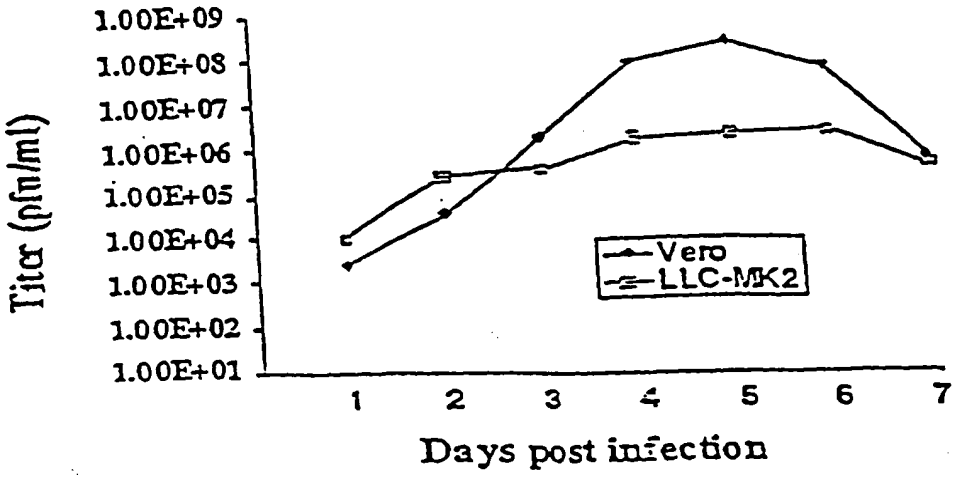
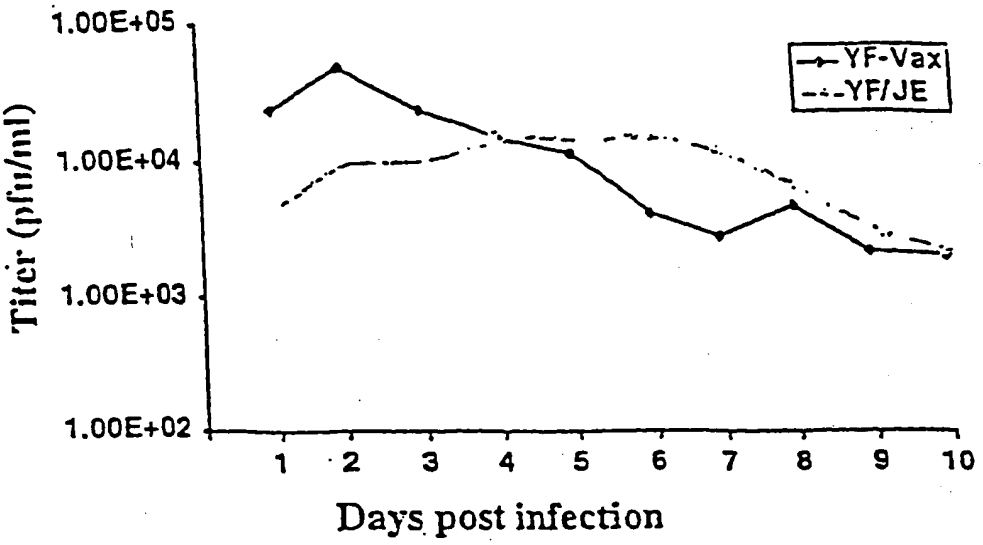

Step Growth Curve
mean
titer
log pfu/ml
7
6
5
4
3
2
1
0
0
20
40
60
80
100
hours post infection
LLC-MK2 cells.
m.o.i. = 0.5 pfu/cell
YF5.2iv
YF/JE SA14-14-2
YF/JE Nakayama
JE SA14-14-2

Fig. 8



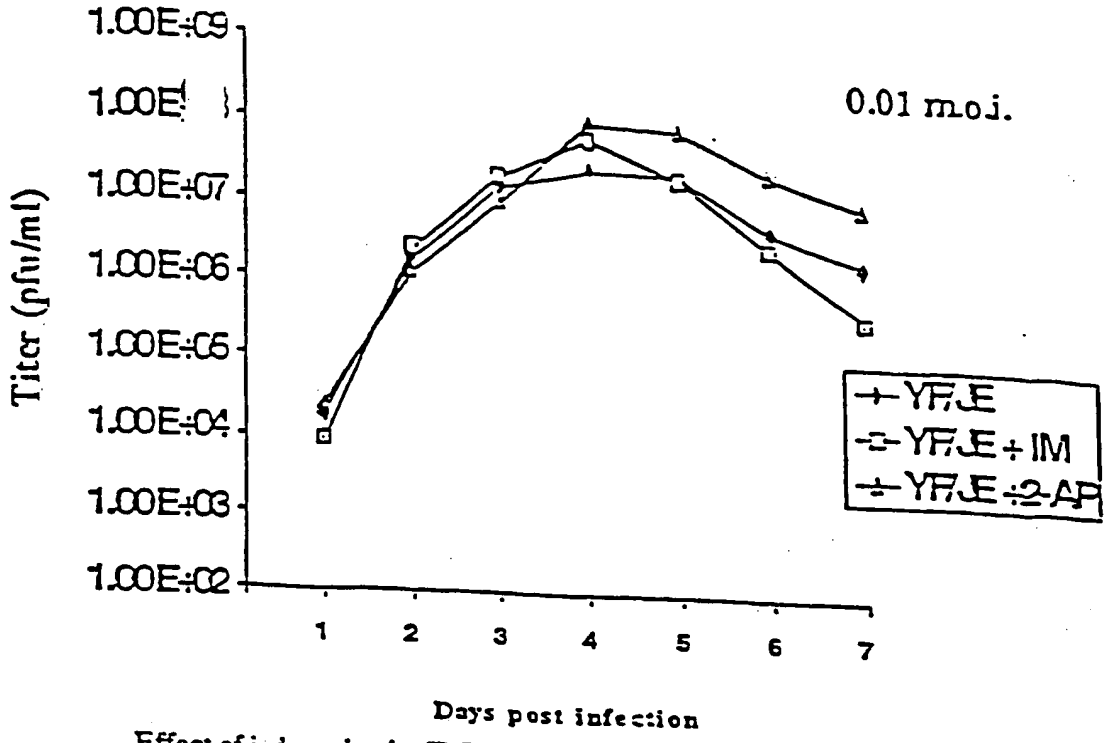
Growth curves of RMS (YF/JE $_{SA14-14-2}$) in Vero and LLC-MK2 cells.

Fig. 9



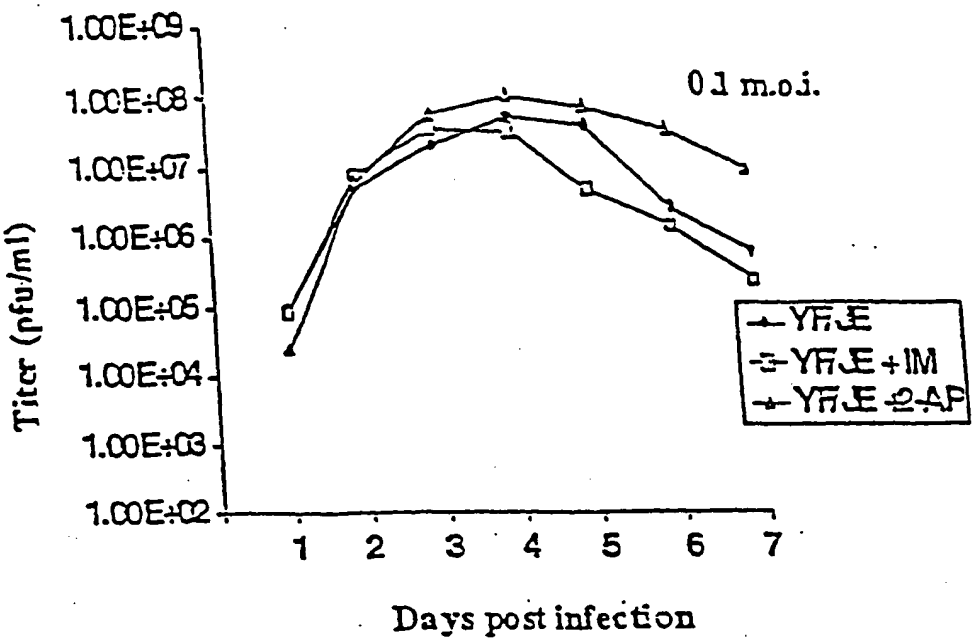
Growth comparison between RMS and YF-Vax in MRC-5 cells.

Fig. 10



YF/JE$_{SA14-14-2}$. Effect of indomethacin (IM) or 2-aminopurine (2-AP) on growth kinetics of (0.01 MOI) in FRhL cells

Fig. 11A

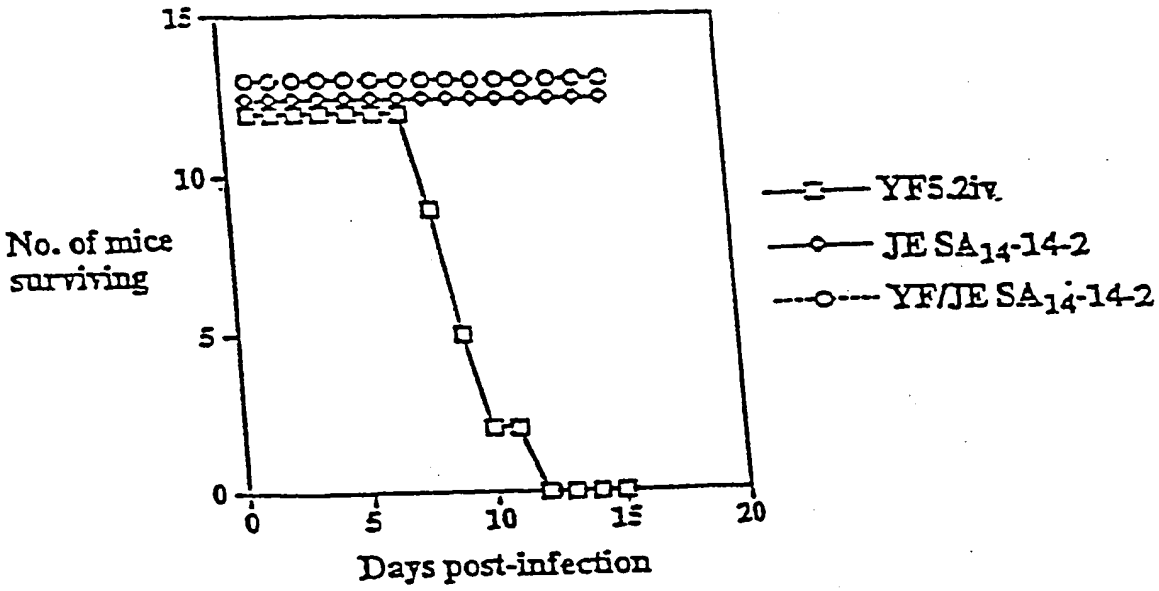


Effect of indomethacin or 2-aminopurine on growth kinetics of YF/JE$_{SA14-14-2}$ (0.1 MOI in FRhL cells.

Fig. 11 B

Mouse neurovirulence analysis

MICE: 4 week old ICR males/females
VIRUS DOSE: 10^4 pfu intracerebrally



Virus	Survival	P
YF5.2iv	0/12 (0%)	-
JE SA_{14}-14-2	12/12 (100%)	<0.001
YF/JE SA_{14}-14-2	13/13 (100%)	<0.001

Fig. 12

Neutralizing antibody response
to YF/JE SA14-14-2 chimeric vaccine
(3-week old mice immunized, samples for testing taken at 6 weeks)

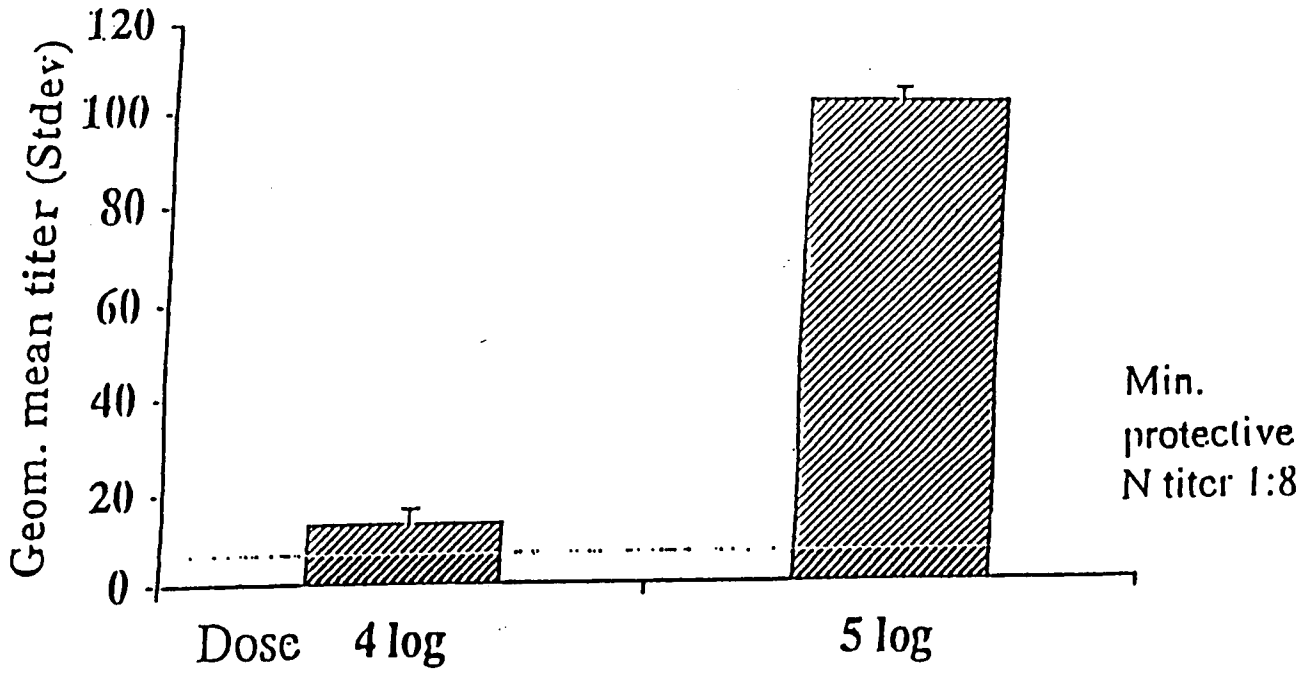


Fig. 13

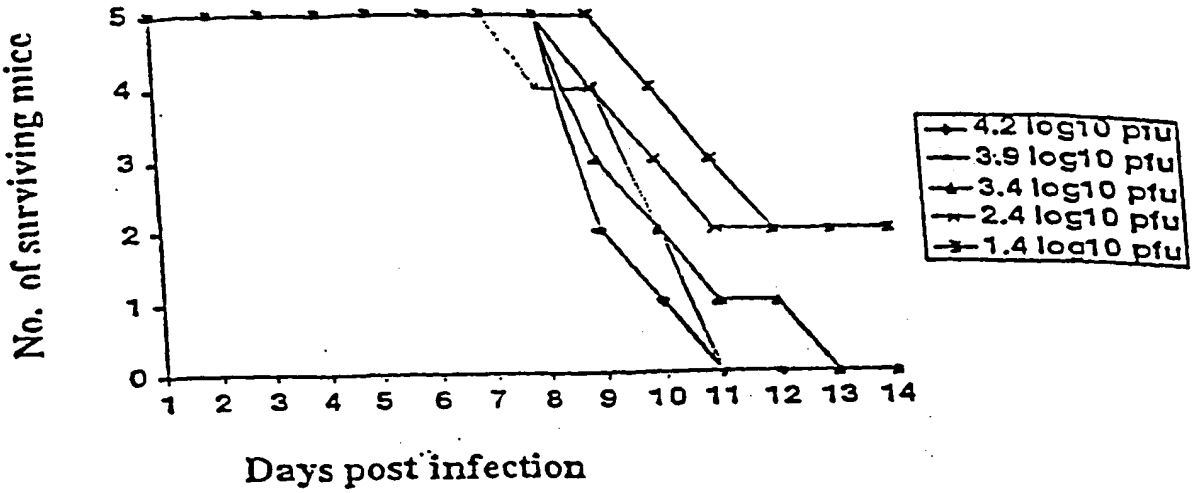


Fig. 14A Neurovirulence testing of YF-Vax in 4-week old ICR mice by the i.c. route.

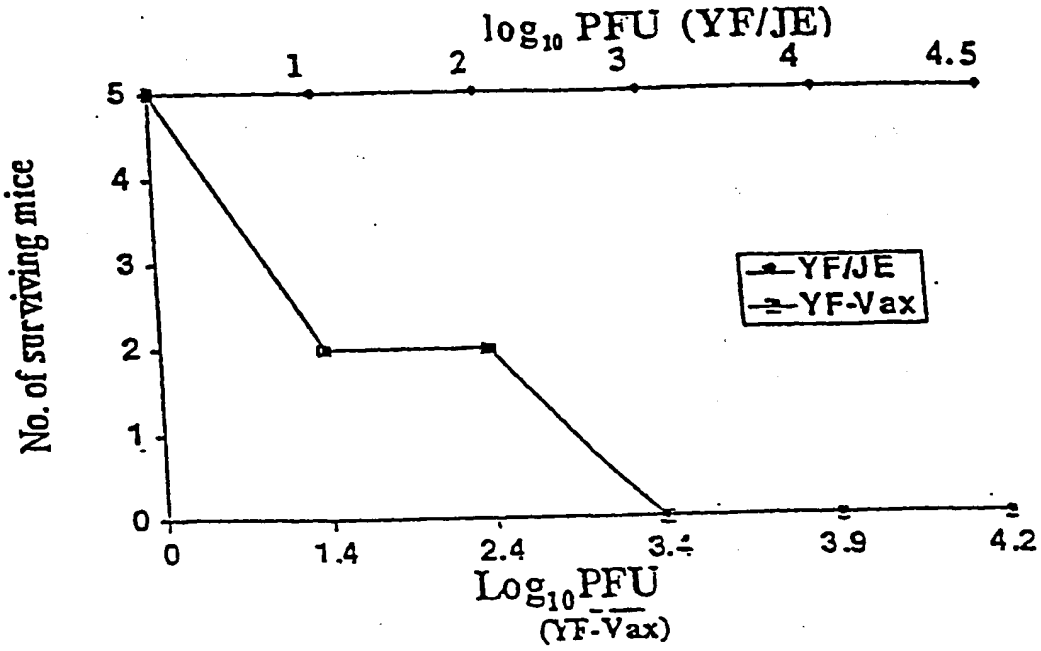


Fig. 14B Neurovirulence testing of $YF/JE_{SA14-14-2}$ in 4-week old ICR mice by I.C. route

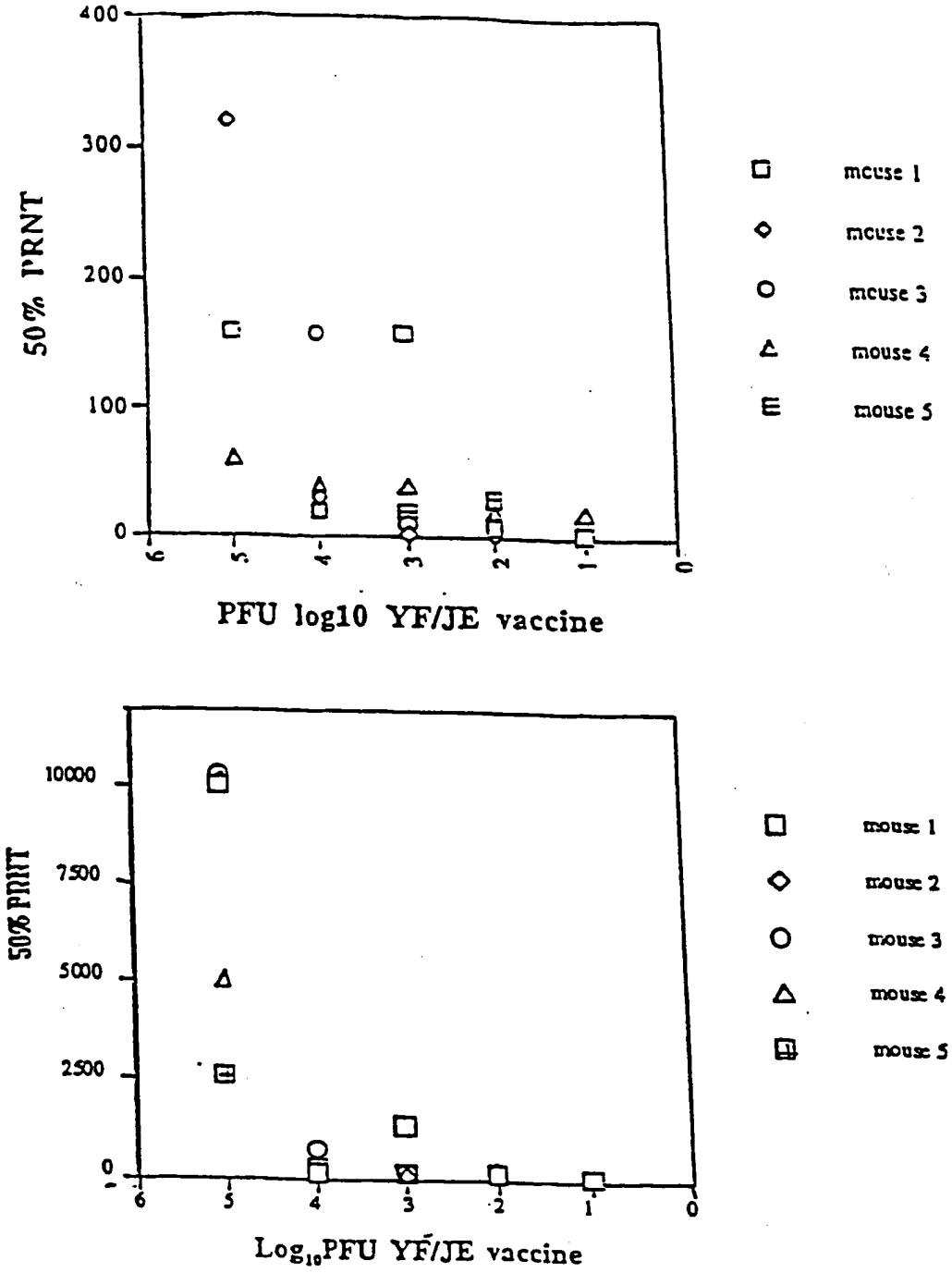


Fig.15 Neutralizing antibody titers in mice inoculated s.c. with graded doses of YF/JE vaccine. TOP: 3 weeks post immunization and BOTTOM: 8 weeks post immunization

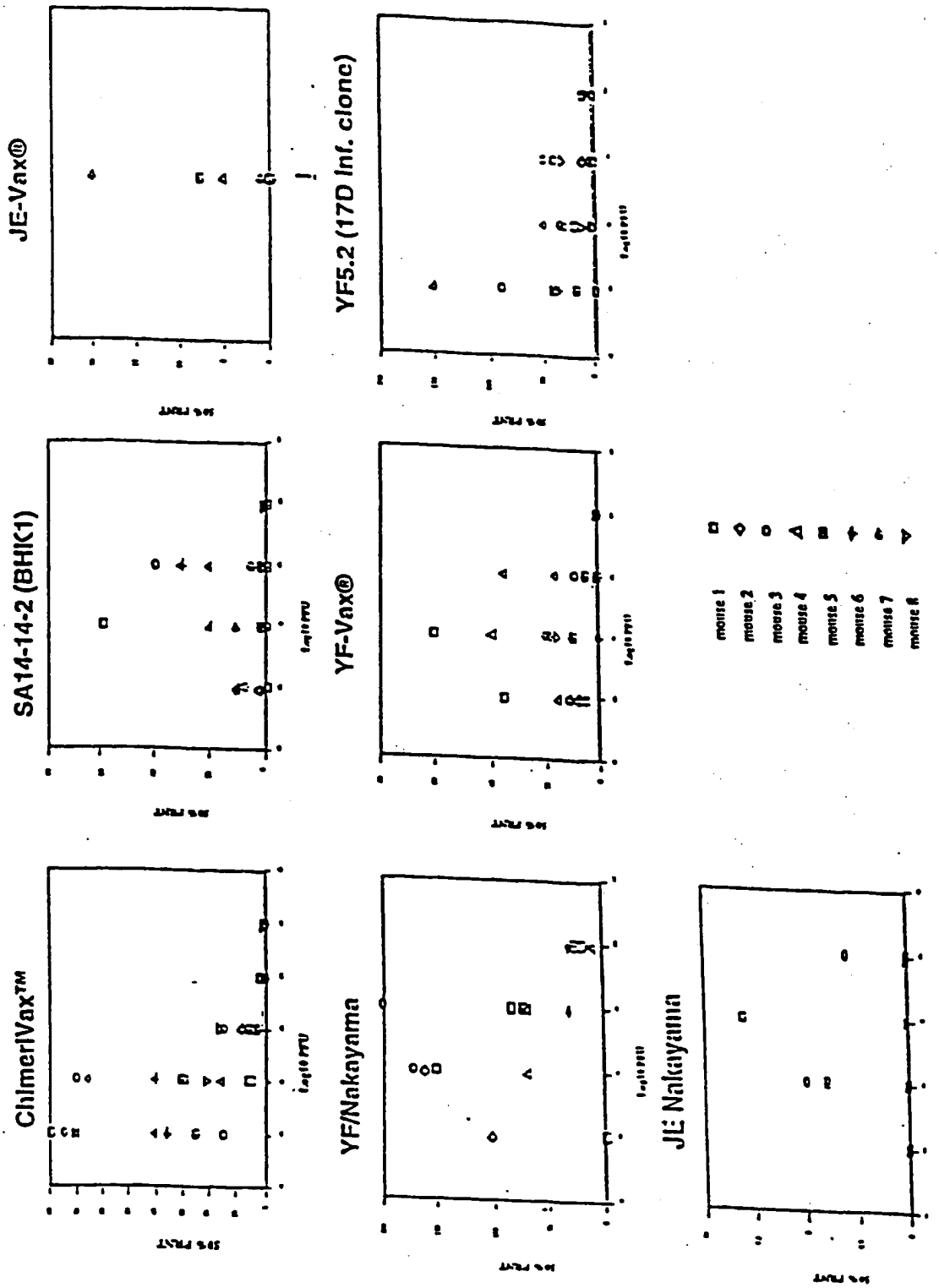
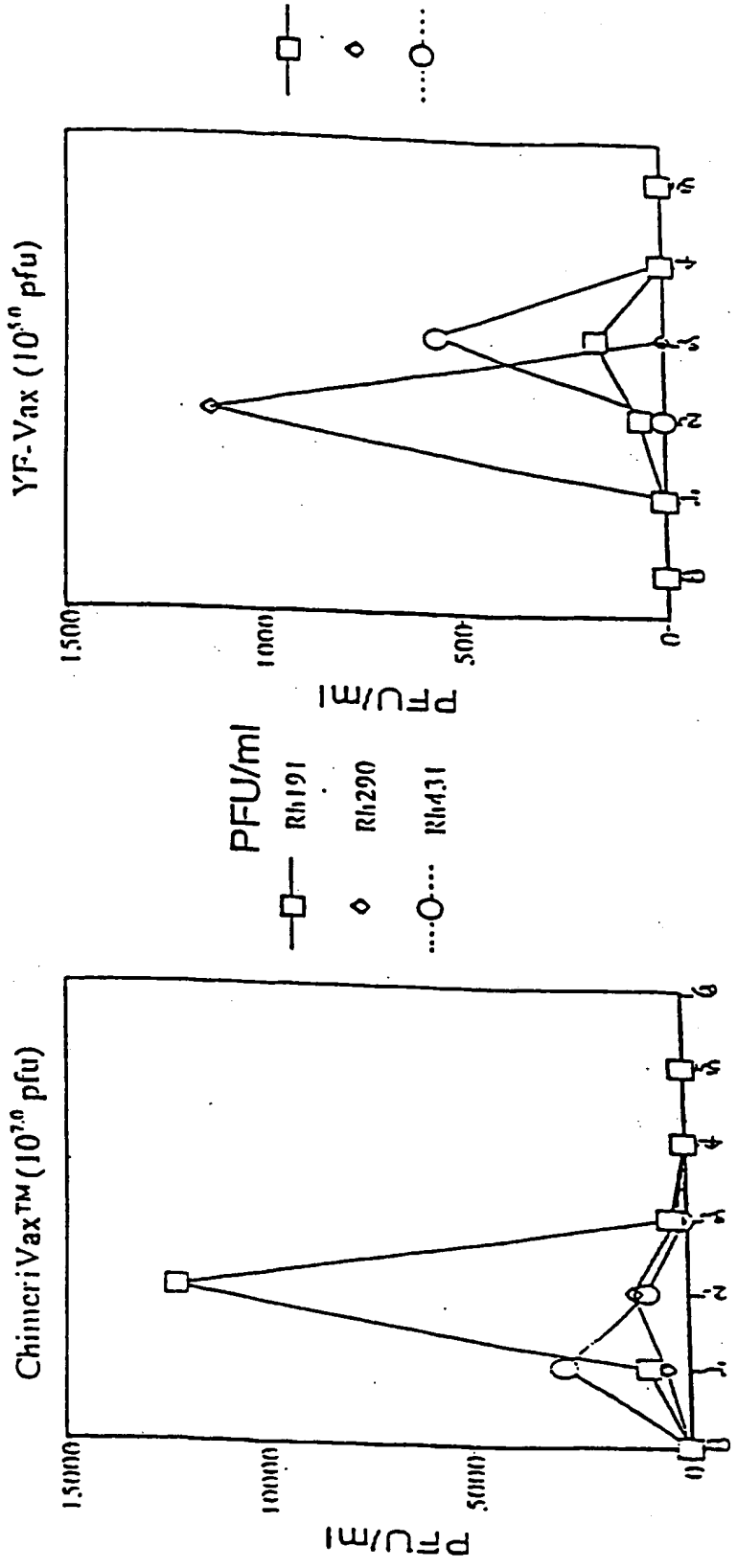
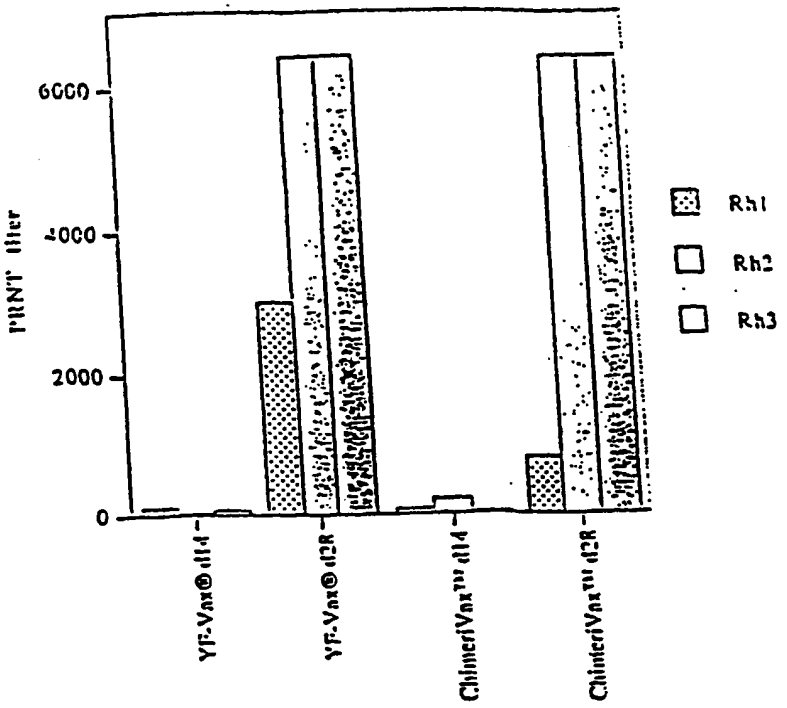


Fig. 16 SEROLOGICAL RESPONSES OF MICE IMMUNIZED WITH A SINGLE DOSE OF LIVE VIRUSES



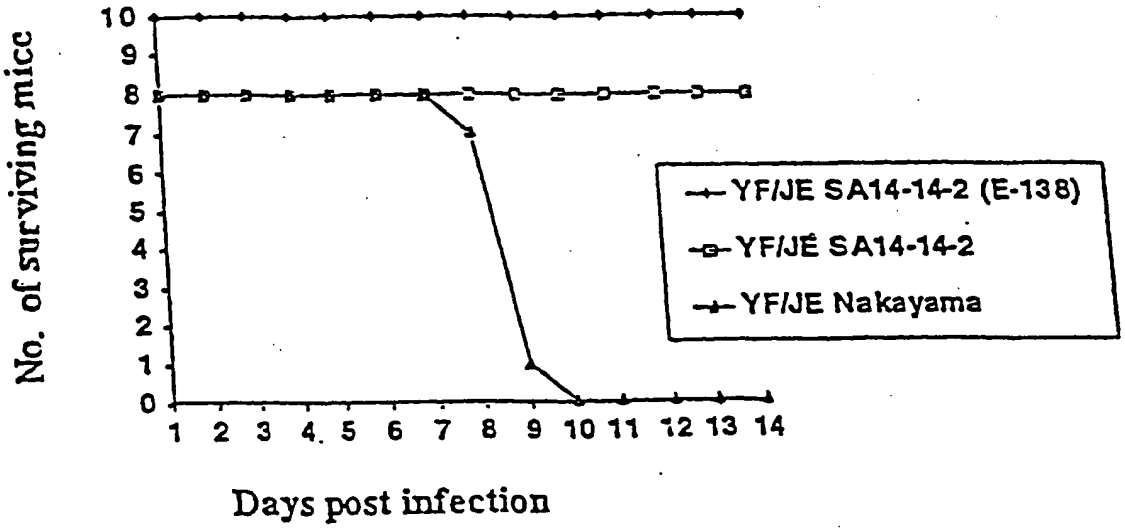
Viremia and GMT of viremia in 3 rhesus monkeys inoculated with ChimeriVax™ or YF-Vax® by the I.C. route.

Fig. 17



Neutralizing antibody titers (50%) in rhesus monkeys 2 and 4 weeks post inoculations with a single dose of vaccines by the I.C. route.

Fig. 18



Mouse neurovirulence testing of YF/JE SA14-14-2 (E-138 K—>E) mutant.

Fig. 19

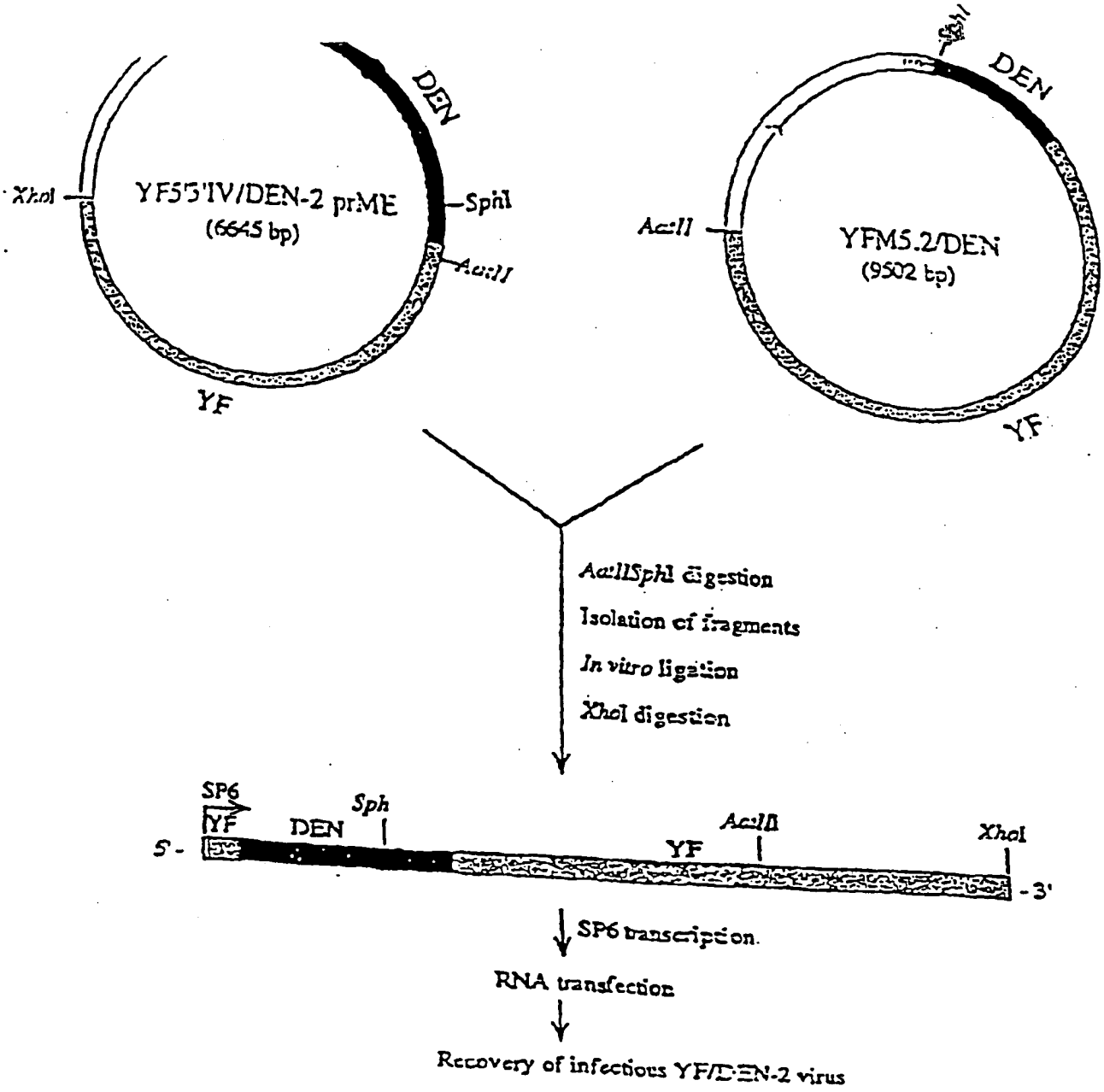

DEN
XhoI
YF5'3'IV/DEN-2 prME
(6645 bp)
SphI
AccII
YF
SphI
DEN
AccII
YFM5.2/DEN
(9502 bp)
YF
AccIISphI digestion
Isolation of fragments
In vitro ligation
XhoI digestion
SP6
YF
DEN
Sph
AccII
XhoI
5'
YF
3'
SP6 transcription
RNA transfection
Recovery of infectious YF/DEN-2 virus

Fig. 20

Structure of modified YF clones expressing E/NS1 intergenic open reading frames

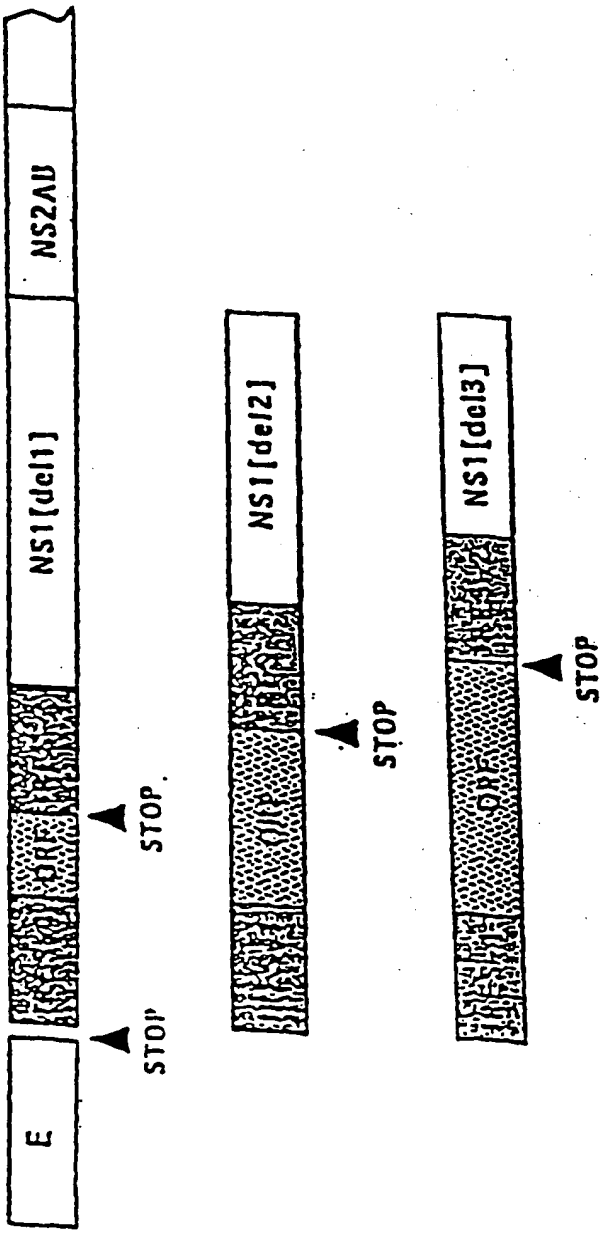
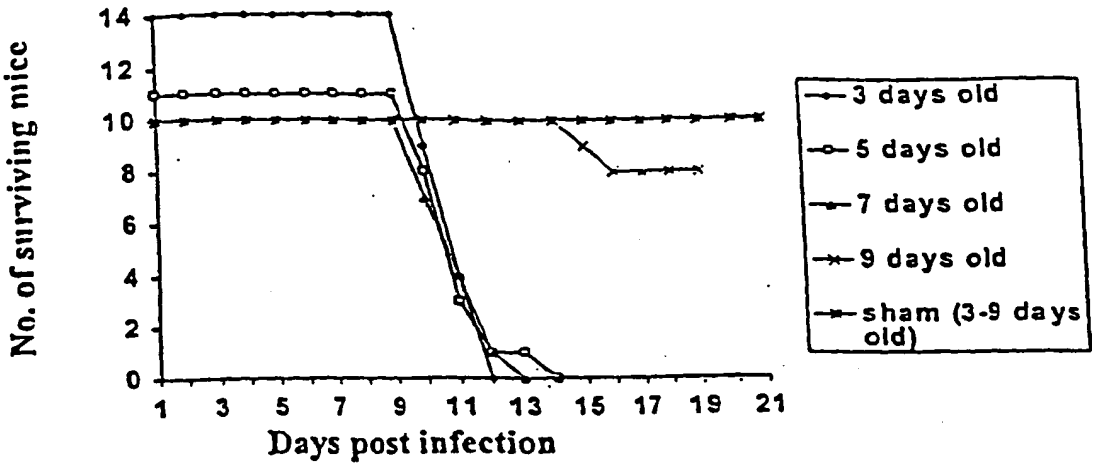
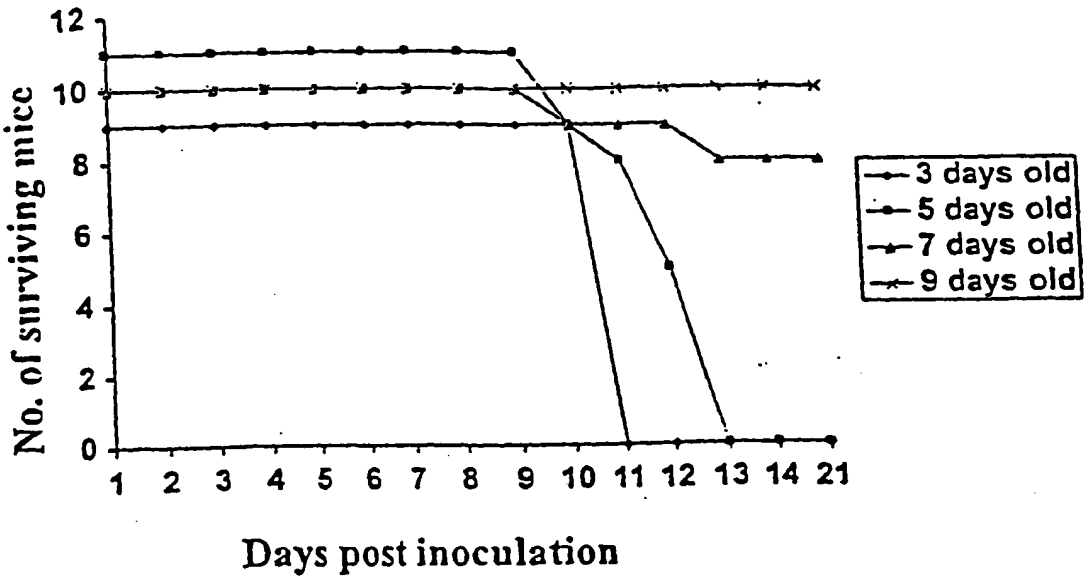


Fig.21



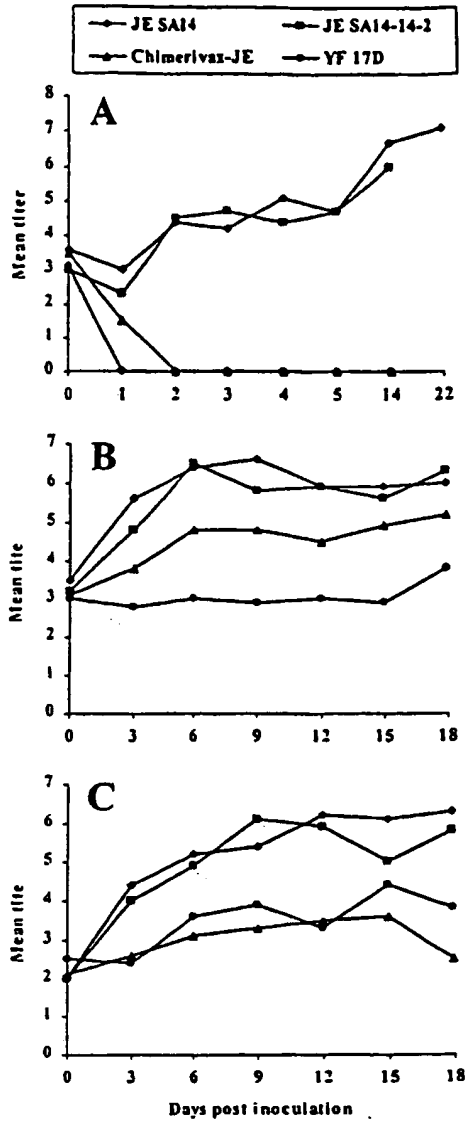
Neurovirulence phenotype of ChimeriVax™-Den2 in outbred (CD-1) suckling mice inoculated by the I.C. route with 10,000 PFU/0.03 ml.

Fig. 22



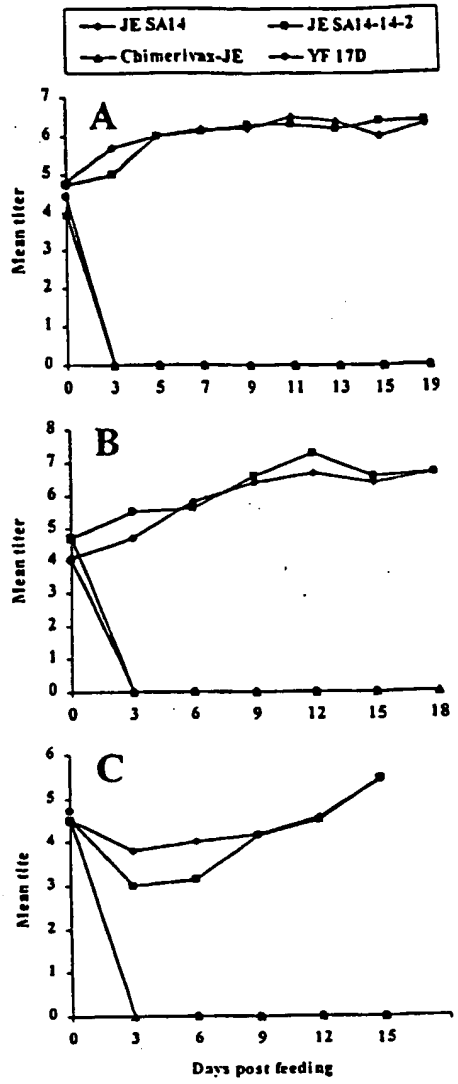
Neurovirulence phenotype of 17D vaccine (YF-Vax®) in outbred (CD-1) suckling mice inoculated by the I.P. route with 1000 PFU/0.02 ml.

Fig. 23



Figs. 24A-C

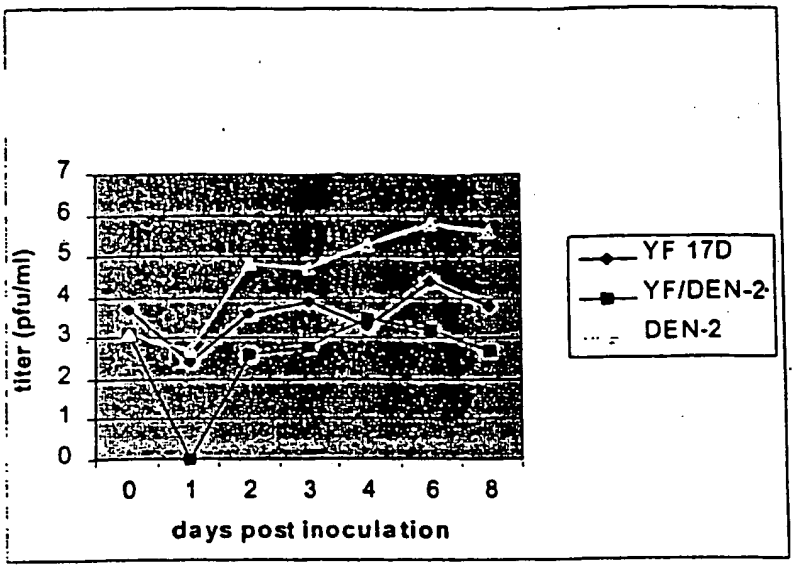
Growth of JE SA14, JE SA14-14-2, ChimeriVax™-JE and YF 17D intrathoracically inoculated mosquitoes. A. *Cx. tritaeniorhynchus* mosquitoes, B. *Ae. albopictus* mosquitoes, C. *Ae. aegypti* mosquitoes. Mean titer = geometric mean of the titers of three individual mosquitoes; $\log_{10}$ pfu/mosquito.



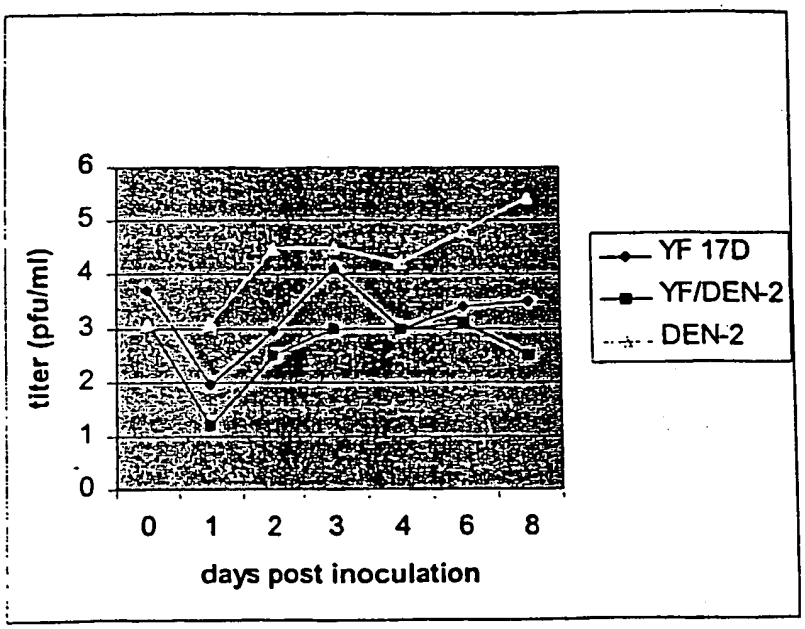
Figs. 25 A-C

Growth of JE SA14, JE SA14-14-2, ChimeriVax™-JE and YF 17D IT orally exposed mosquitoes. A. *Cx. tritaeniorhynchus* mosquitoes, B. *Ae. albopictus* mosquitoes, C. *Ae. aegypti* mosquitoes. Mean titer = geometric mean of the titers of three individual mosquitoes; $\log_{10}$ pfu/mosquito.

A



B



. Growth of virus in IT inoculated *Ae. aegypti* (A) and *Ae. albopictus* (B) mosquitoes.

Figs. 26 A and B

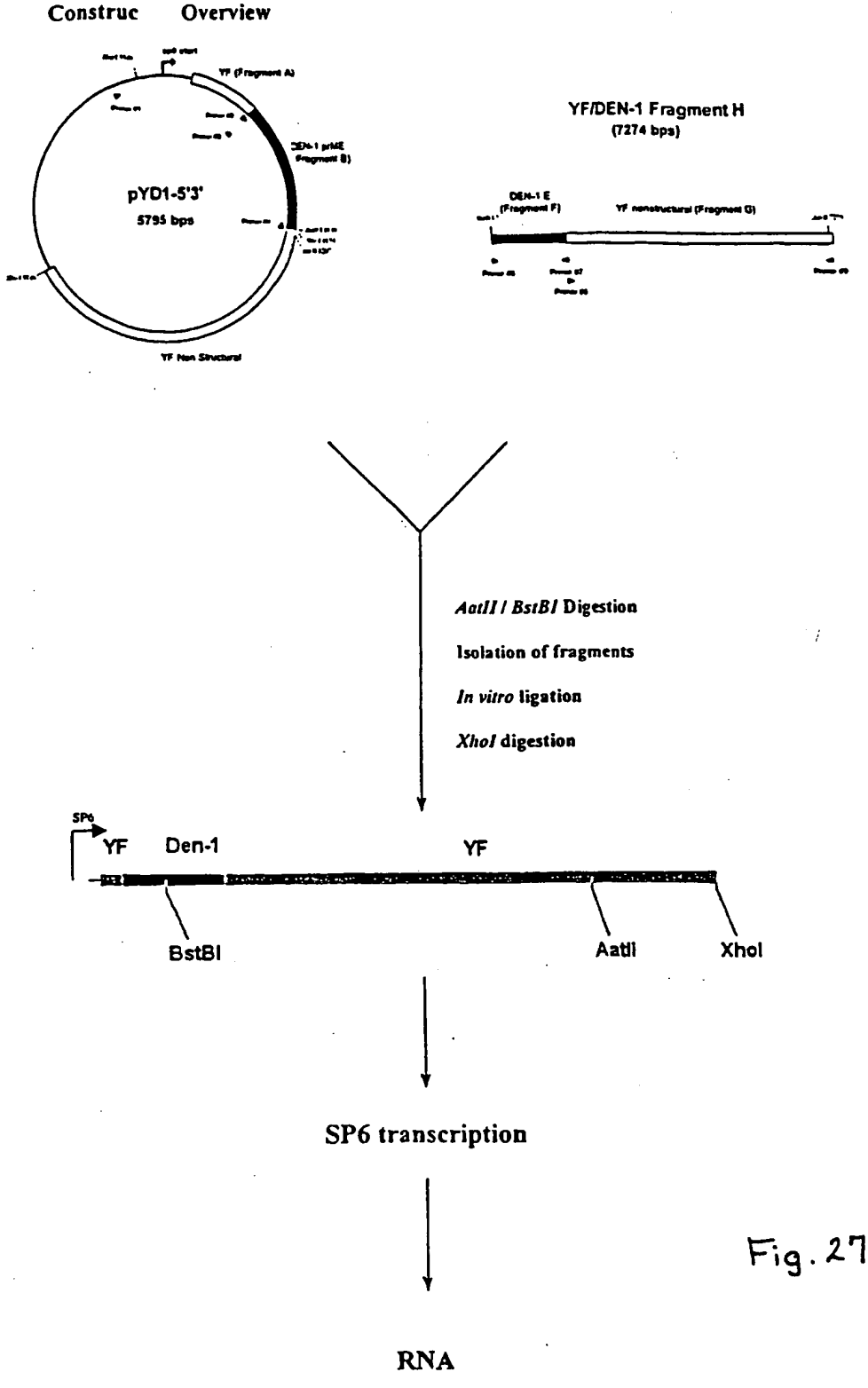
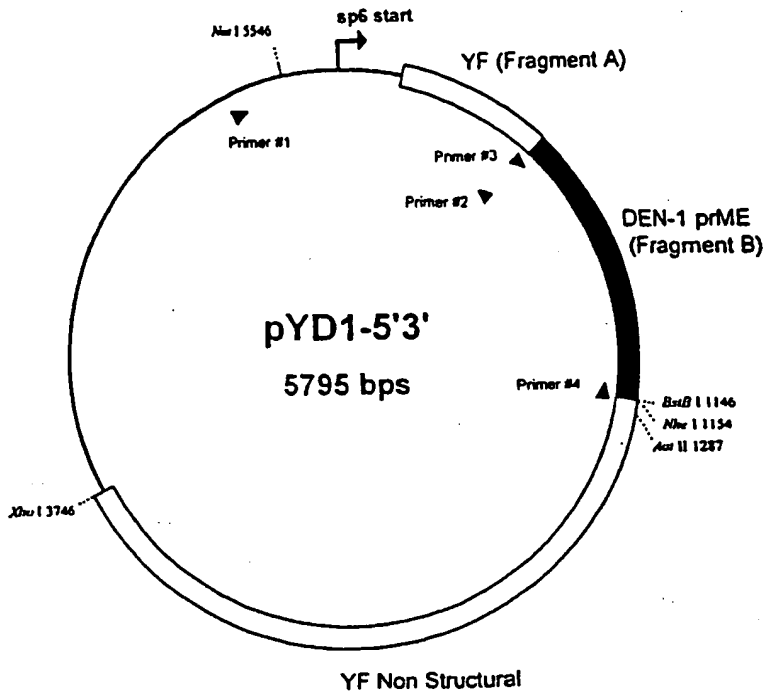

Construc Overview
pYD1-5'3'
5795 bps
YF Non Structural
YF/DEN-1 Fragment H
(7274 bps)
DEN-1 E
(Fragment F)
YF nonstructural (Fragment G)
AatII / BstBI Digestion
Isolation of fragments
In vitro ligation
XhoI digestion
SP6
YF
Den-1
YF
BstBI
AatII
XhoI
SP6 transcription
RNA

Fig. 27

Plasmid and Fragment Maps



YF/DEN-1 Fragment H
(7274 bps)

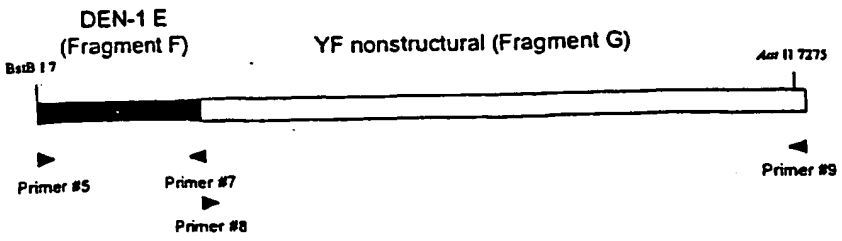


Fig. 28

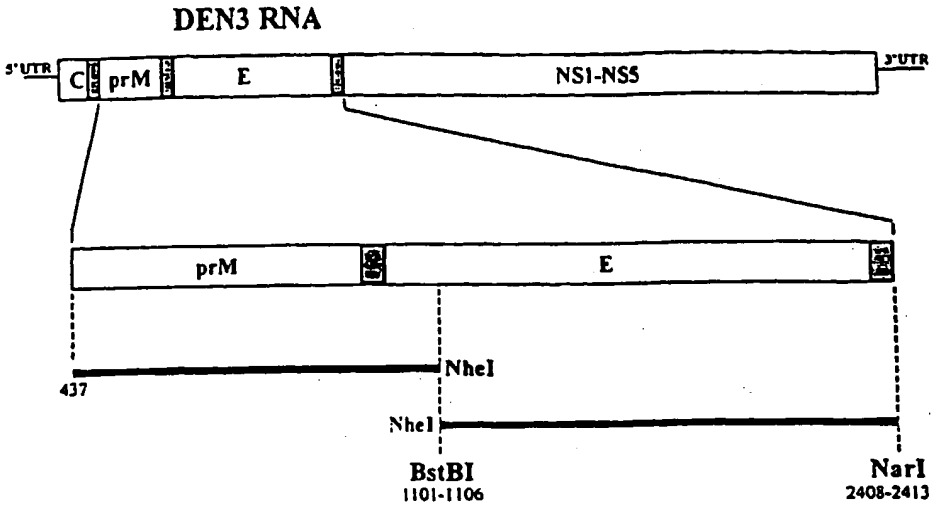


Figure 1. RT-PCR amplification of the prM-E region of the PaH881/88 DEN3 virus genome. The virus genome is shown on the top diagram. Regions encoding hydrophobic signals for corresponding downstream proteins are shadowed. The prM-E region was amplified in two fragments (black solid lines). Restriction sites introduced for subsequent in-frame in vitro ligation into YF backbone (BstBI and NarI) and cloning (NheI) are indicated.

Fig. 29

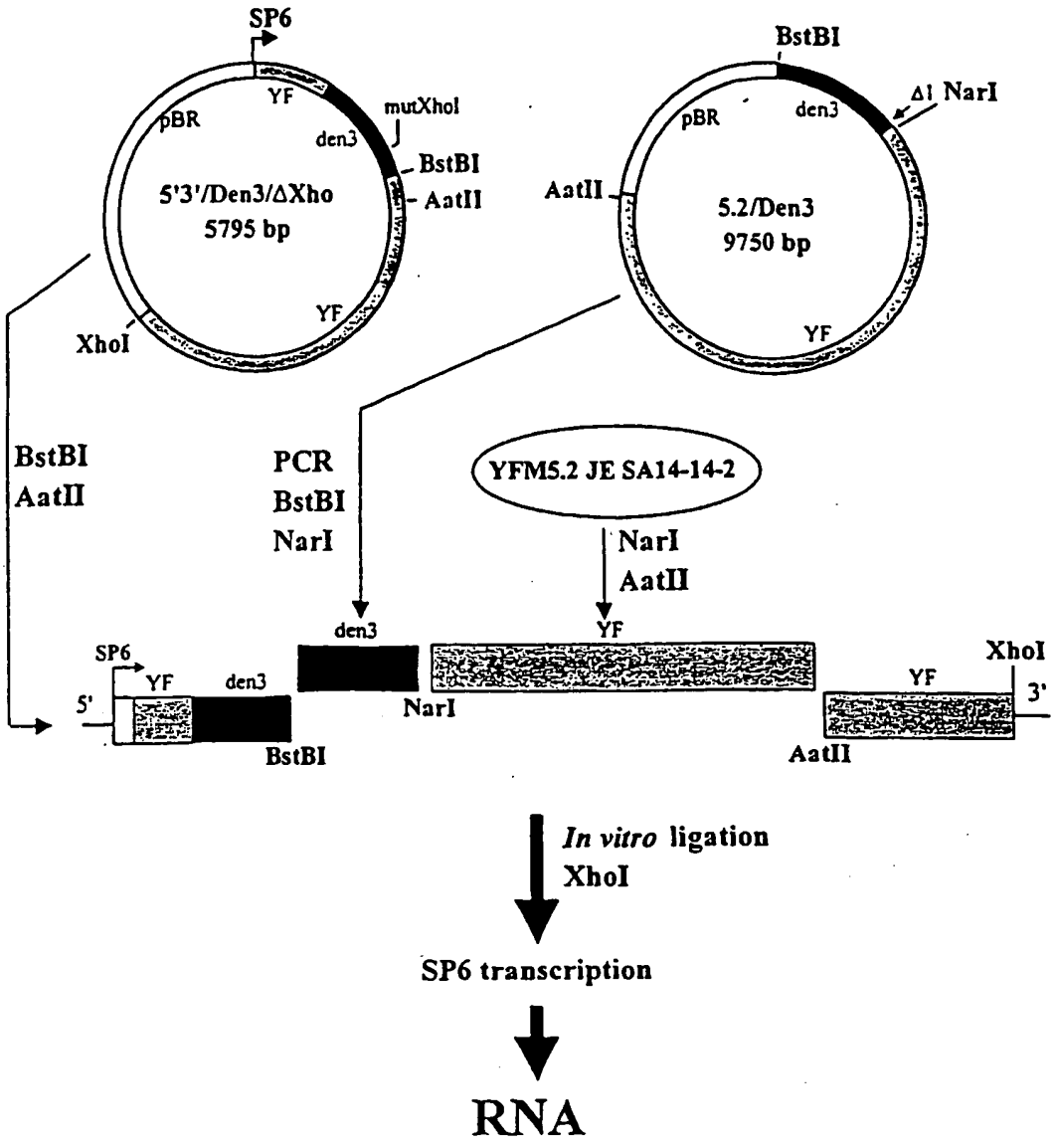

SP6
YF
mutXhoI
pBR
den3
BstBI
AatII
5'3'/Den3/ΔXho
5795 bp
YF
XhoI
BstBI
AatII
BstBI
Δ1 NarI
pBR
den3
AatII
5.2/Den3
9750 bp
YF
BstBI
AatII
PCR
BstBI
NarI
YFM5.2 JE SA14-14-2
NarI
AatII
SP6
YF
den3
5'
BstBI
den3
NarI
YF
YF
XhoI
3'
AatII
In vitro ligation
XhoI
SP6 transcription
RNA

Fig. 30

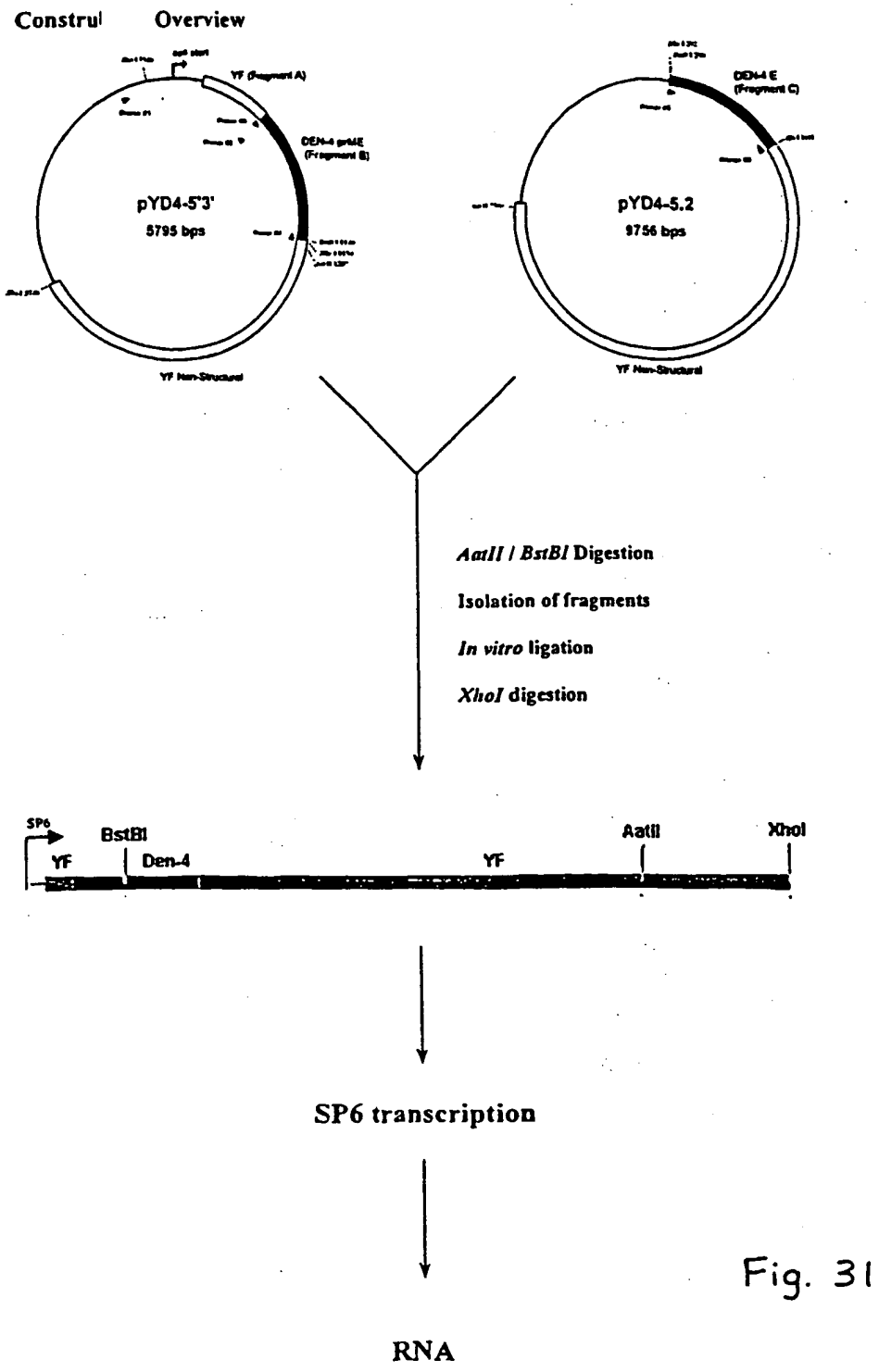

Construl Overview
pYD4-5'3'
5795 bps
pYD4-5.2
9756 bps
AatII / BstBI Digestion
Isolation of fragments
In vitro ligation
XhoI digestion
SP6
BstBI
AatII
XhoI
YF
Den-4
YF
SP6 transcription
RNA

Fig. 31

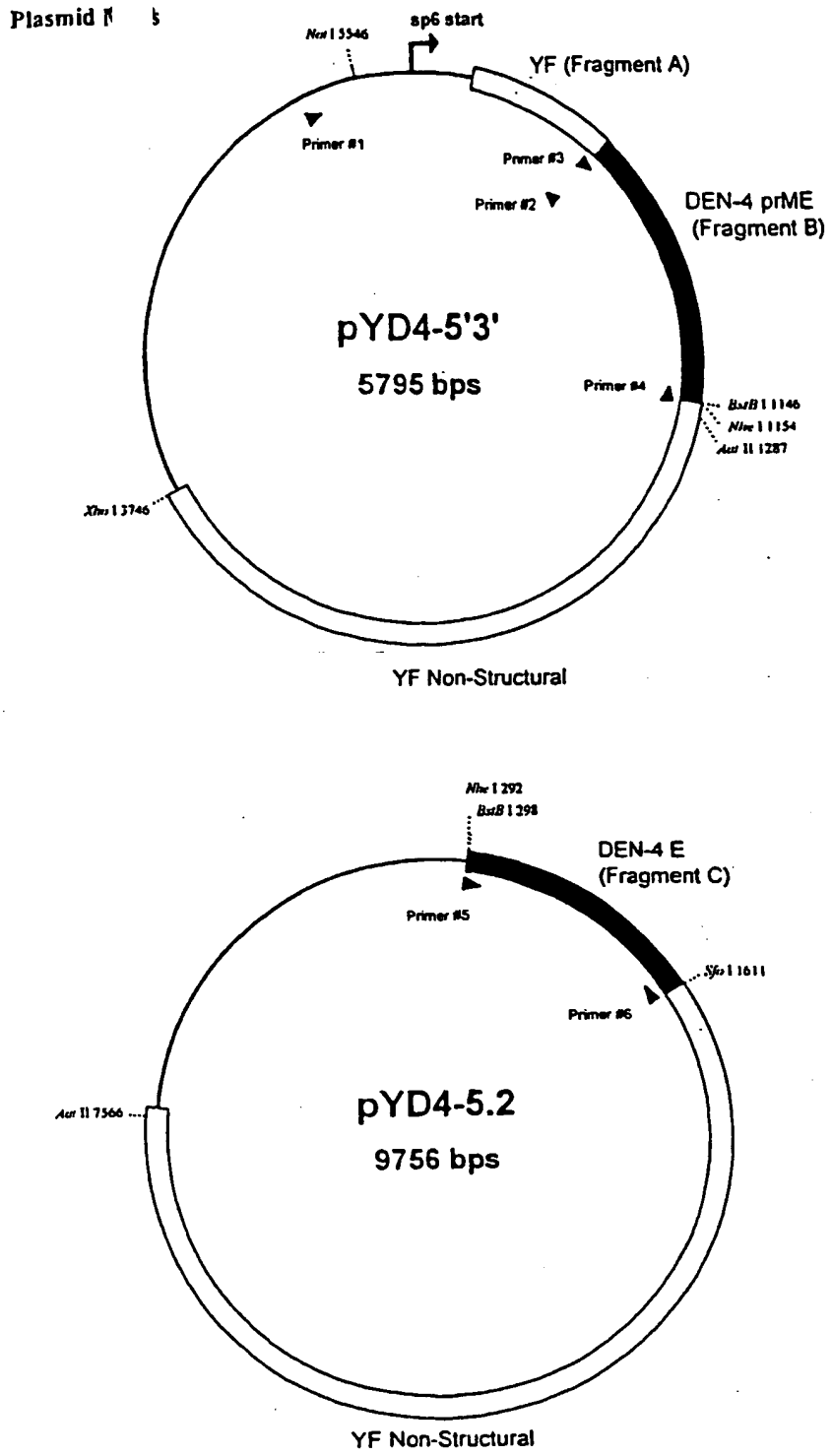

Plasmid
sp6 start
YF (Fragment A)
Primer #1
Primer #3
Primer #2
DEN-4 prME
(Fragment B)
pYD4-5'3'
5795 bps
Primer #4
YF Non-Structural
Nhe I 292
BstB I 298
DEN-4 E
(Fragment C)
Primer #5
Sfo I 1611
Primer #6
pYD4-5.2
9756 bps
YF Non-Structural

Fig. 32

SEQUENCE LISTING

<110> OraVax, Inc.

<120> Chimeric Flavivirus Vaccines

<130> 06132/033WO4

<150> US 09/452,638
<151> 1999-12-01

<160> 85

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<210> 46
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gccgccggcg ccaactgtga aacctagaaa cacag 35

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<400> 49
atttaggtga cactatagag taaatcctgt gtgctaatt 39

<210> 50
<211> 1983

<212> DNA
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<220>
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<222> (1)...(1983)

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Phe His Leu Thr Thr Arg Asn Gly Glu Pro His Met Ile Val Ser Arg
 1               5                  10                  15

caa gag aaa ggg aaa agt ctt ttg ttt aaa aca gag gat ggc gtg aac   96
Gln Glu Lys Gly Lys Ser Leu Leu Phe Lys Thr Glu Asp Gly Val Asn
            20                  25                  30

atg tgc acc ctc atg gcc atg gac ctt ggt gaa ttg tgt gaa gac aca  144
Met Cys Thr Leu Met Ala Met Asp Leu Gly Glu Leu Cys Glu Asp Thr
        35                  40                  45

atc acg tac aag tgt ccc ctt ctc agg cag aat gag cca gaa gac ata  192
Ile Thr Tyr Lys Cys Pro Leu Leu Arg Gln Asn Glu Pro Glu Asp Ile
    50                  55                  60

gac tgc tgg tgc aac tcc acg tcc acg tgg gta acc tat ggg act tgt  240
Asp Cys Trp Cys Asn Ser Thr Ser Thr Trp Val Thr Tyr Gly Thr Cys
65                  70                  75                  80

acc acc acg gga gaa cat aga aga gaa aaa aga tca gtg gca ctc gtt  288
Thr Thr Thr Gly Glu His Arg Arg Glu Lys Arg Ser Val Ala Leu Val
                85                  90                  95

cca cat gtg gga atg gga ctg gag acg cga act gaa aca tgg atg tca  336
Pro His Val Gly Met Gly Leu Glu Thr Arg Thr Glu Thr Trp Met Ser
            100                 105                 110

tca gaa ggg gct tgg aaa cat gcc cag aga att gaa att tgg atc ctg  384
Ser Glu Gly Ala Trp Lys His Ala Gln Arg Ile Glu Ile Trp Ile Leu
        115                 120                 125

aga cat cca ggc ttc acc ata atg gca gca atc ctg gca tac acc ata  432
Arg His Pro Gly Phe Thr Ile Met Ala Ala Ile Leu Ala Tyr Thr Ile
    130                 135                 140

ggg acg aca cat ttc cag aga gca ctg att ttc atc tta ctg aca gct  480
Gly Thr Thr His Phe Gln Arg Ala Leu Ile Phe Ile Leu Leu Thr Ala
145                 150                 155                 160

gtc gct cct tca atg aca atg cgt tgc ata gga ata tca aat aga gac  528
Val Ala Pro Ser Met Thr Met Arg Cys Ile Gly Ile Ser Asn Arg Asp
                165                 170                 175

ttt gta gaa ggg gtt tca gga gga agc tgg gtt gac ata gtc tta gaa  576
Phe Val Glu Gly Val Ser Gly Gly Ser Trp Val Asp Ile Val Leu Glu
            180                 185                 190

cat gga agc tgt gtg acg acg atg gca aaa aac aaa cca aca ttg gat  624
His Gly Ser Cys Val Thr Thr Met Ala Lys Asn Lys Pro Thr Leu Asp
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        195                 200                 205

ttt gaa ctg ata aaa aca gaa gcc aaa cag cct gcc acc cta agg aag   672
Phe Glu Leu Ile Lys Thr Glu Ala Lys Gln Pro Ala Thr Leu Arg Lys
    210                 215                 220

tac tgt ata gag gca aag cta acc aac aca aca aca gaa tct cgt tgc   720
Tyr Cys Ile Glu Ala Lys Leu Thr Asn Thr Thr Thr Glu Ser Arg Cys
225                 230                 235                 240

cca aca caa ggg gaa ccc agc cta aat gaa gag cag gat aaa agg ttc   768
Pro Thr Gln Gly Glu Pro Ser Leu Asn Glu Glu Gln Asp Lys Arg Phe
                245                 250                 255

gtc tgc aaa cac tcc atg gta gac aga gga tgg gga aat gga tgt gga   816
Val Cys Lys His Ser Met Val Asp Arg Gly Trp Gly Asn Gly Cys Gly
            260                 265                 270

tta ttt gga aag gga ggc att gtg acc tgt gct atg ttc aca tgc aaa   864
Leu Phe Gly Lys Gly Gly Ile Val Thr Cys Ala Met Phe Thr Cys Lys
        275                 280                 285

aag aac atg gag gga aaa gtt gtg cag cca gaa aac ttg gaa tac acc   912
Lys Asn Met Glu Gly Lys Val Val Gln Pro Glu Asn Leu Glu Tyr Thr
    290                 295                 300

att gtg gta aca ccc cac tca ggg gaa gag cat gcg gtc gga aat gac   960
Ile Val Val Thr Pro His Ser Gly Glu Glu His Ala Val Gly Asn Asp
305                 310                 315                 320

aca gga aaa cat ggc aag gaa atc aaa gta aca cca cag agt tcc atc  1008
Thr Gly Lys His Gly Lys Glu Ile Lys Val Thr Pro Gln Ser Ser Ile
                325                 330                 335

aca gaa gca gaa ttg aca ggt tat ggc act gtc acg atg gag tgc tct  1056
Thr Glu Ala Glu Leu Thr Gly Tyr Gly Thr Val Thr Met Glu Cys Ser
            340                 345                 350

ccg aga aca ggc ctc gac ttc aat gag atg gtg ttg ctg cag atg gaa  1104
Pro Arg Thr Gly Leu Asp Phe Asn Glu Met Val Leu Leu Gln Met Glu
        355                 360                 365

aat aaa gct tgg ctg gtg cat agg caa tgg ttc cta gac ctg ccg tta  1152
Asn Lys Ala Trp Leu Val His Arg Gln Trp Phe Leu Asp Leu Pro Leu
    370                 375                 380

cca tgg ctg ccc gga gcg gac aca caa ggg tca aat tgg ata caa aaa  1200
Pro Trp Leu Pro Gly Ala Asp Thr Gln Gly Ser Asn Trp Ile Gln Lys
385                 390                 395                 400

gaa aca ttg gtc act ttc aaa aat cct cat gcg aag aaa cag gat gtt  1248
Glu Thr Leu Val Thr Phe Lys Asn Pro His Ala Lys Lys Gln Asp Val
                405                 410                 415

gtt gtt tta gga tcc caa gaa ggg gcc atg cac aca gca ctc aca ggg  1296
Val Val Leu Gly Ser Gln Glu Gly Ala Met His Thr Ala Leu Thr Gly
            420                 425                 430
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gcc aca gaa atc caa atg tca tca gga aac tta ctc ttc aca gga cat   1344
Ala Thr Glu Ile Gln Met Ser Ser Gly Asn Leu Leu Phe Thr Gly His
        435                 440                 445

ctc aag tgc agg ctg aga atg gac aag cta cag ctc aaa gga atg tca   1392
Leu Lys Cys Arg Leu Arg Met Asp Lys Leu Gln Leu Lys Gly Met Ser
    450                 455                 460

tac tct atg tgc aca gga aag ttt aaa gtt gtg aag gaa ata gca gaa   1440
Tyr Ser Met Cys Thr Gly Lys Phe Lys Val Val Lys Glu Ile Ala Glu
465                 470                 475                 480

aca caa cat gga aca ata gtt atc agg gtg cag tat gaa ggg gac ggc   1488
Thr Gln His Gly Thr Ile Val Ile Arg Val Gln Tyr Glu Gly Asp Gly
                485                 490                 495

tct cca tgt aaa atc cct ttt gag ata atg gat ttg gaa aaa aga cat   1536
Ser Pro Cys Lys Ile Pro Phe Glu Ile Met Asp Leu Glu Lys Arg His
            500                 505                 510

gtc tta ggt cgc ctg atc aca gtc aac cca att gtg aca gaa aaa gat   1584
Val Leu Gly Arg Leu Ile Thr Val Asn Pro Ile Val Thr Glu Lys Asp
        515                 520                 525

agc cca gtc aac ata gaa gca gaa cct cca ttc gga gac agc tac atc   1632
Ser Pro Val Asn Ile Glu Ala Glu Pro Pro Phe Gly Asp Ser Tyr Ile
    530                 535                 540

atc ata gga gta gag ccg gga caa ctg aag ctc aac tgg ttt aag aaa   1680
Ile Ile Gly Val Glu Pro Gly Gln Leu Lys Leu Asn Trp Phe Lys Lys
545                 550                 555                 560

gga agt tct atc ggc caa atg ttt gag aca aca atg agg ggg gcg aag   1728
Gly Ser Ser Ile Gly Gln Met Phe Glu Thr Thr Met Arg Gly Ala Lys
                565                 570                 575

aga atg gcc att ttg ggt gac aca gcc tgg gat ttt gga tcc ctg gga   1776
Arg Met Ala Ile Leu Gly Asp Thr Ala Trp Asp Phe Gly Ser Leu Gly
            580                 585                 590

gga gtg ttt aca tct ata gga aaa gcc ctc cac caa gtc ttt gga gca   1824
Gly Val Phe Thr Ser Ile Gly Lys Ala Leu His Gln Val Phe Gly Ala
        595                 600                 605

atc tat gga gct gcc ttc agt ggg gtc tca tgg act atg aaa atc ctc   1872
Ile Tyr Gly Ala Ala Phe Ser Gly Val Ser Trp Thr Met Lys Ile Leu
    610                 615                 620

ata gga gtc att atc aca tgg ata gga atg aat tca cgc agc acc tca   1920
Ile Gly Val Ile Ile Thr Trp Ile Gly Met Asn Ser Arg Ser Thr Ser
625                 630                 635                 640

ctg tct gtg tca cta gta ttg gtg gga gtc gtg acg ctg tat ttg gga   1968
Leu Ser Val Ser Leu Val Leu Val Gly Val Val Thr Leu Tyr Leu Gly
                645                 650                 655

gtt atg gtg ggc gcc                                              1983
Val Met Val Gly Ala
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660

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<213> Dengue-2 virus

<400> 51

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Gln Glu Lys Gly Lys Ser Leu Leu Phe Lys Thr Glu Asp Gly Val Asn
            20                  25                  30
Met Cys Thr Leu Met Ala Met Asp Leu Gly Glu Leu Cys Glu Asp Thr
        35                  40                  45
Ile Thr Tyr Lys Cys Pro Leu Leu Arg Gln Asn Glu Pro Glu Asp Ile
    50                  55                  60
Asp Cys Trp Cys Asn Ser Thr Ser Thr Trp Val Thr Tyr Gly Thr Cys
65                  70                  75                  80
Thr Thr Thr Gly Glu His Arg Arg Glu Lys Arg Ser Val Ala Leu Val
                85                  90                  95
Pro His Val Gly Met Gly Leu Glu Thr Arg Thr Glu Thr Trp Met Ser
            100                 105                 110
Ser Glu Gly Ala Trp Lys His Ala Gln Arg Ile Glu Ile Trp Ile Leu
        115                 120                 125
Arg His Pro Gly Phe Thr Ile Met Ala Ala Ile Leu Ala Tyr Thr Ile
    130                 135                 140
Gly Thr Thr His Phe Gln Arg Ala Leu Ile Phe Ile Leu Leu Thr Ala
145                 150                 155                 160
Val Ala Pro Ser Met Thr Met Arg Cys Ile Gly Ile Ser Asn Arg Asp
                165                 170                 175
Phe Val Glu Gly Val Ser Gly Gly Ser Trp Val Asp Ile Val Leu Glu
            180                 185                 190
His Gly Ser Cys Val Thr Thr Met Ala Lys Asn Lys Pro Thr Leu Asp
        195                 200                 205
Phe Glu Leu Ile Lys Thr Glu Ala Lys Gln Pro Ala Thr Leu Arg Lys
    210                 215                 220
Tyr Cys Ile Glu Ala Lys Leu Thr Asn Thr Thr Thr Glu Ser Arg Cys
225                 230                 235                 240
Pro Thr Gln Gly Glu Pro Ser Leu Asn Glu Glu Gln Asp Lys Arg Phe
                245                 250                 255
Val Cys Lys His Ser Met Val Asp Arg Gly Trp Gly Asn Gly Cys Gly
            260                 265                 270
Leu Phe Gly Lys Gly Gly Ile Val Thr Cys Ala Met Phe Thr Cys Lys
        275                 280                 285
Lys Asn Met Glu Gly Lys Val Val Gln Pro Glu Asn Leu Glu Tyr Thr
    290                 295                 300
Ile Val Val Thr Pro His Ser Gly Glu Glu His Ala Val Gly Asn Asp
305                 310                 315                 320
Thr Gly Lys His Gly Lys Glu Ile Lys Val Thr Pro Gln Ser Ser Ile
                325                 330                 335
Thr Glu Ala Glu Leu Thr Gly Tyr Gly Thr Val Thr Met Glu Cys Ser
            340                 345                 350
Pro Arg Thr Gly Leu Asp Phe Asn Glu Met Val Leu Leu Gln Met Glu
        355                 360                 365
Asn Lys Ala Trp Leu Val His Arg Gln Trp Phe Leu Asp Leu Pro Leu
    370                 375                 380
Pro Trp Leu Pro Gly Ala Asp Thr Gln Gly Ser Asn Trp Ile Gln Lys
```

```
385                 390                 395                 400
Glu Thr Leu Val Thr Phe Lys Asn Pro His Ala Lys Lys Gln Asp Val
                405                 410                 415
Val Val Leu Gly Ser Gln Glu Gly Ala Met His Thr Ala Leu Thr Gly
            420                 425                 430
Ala Thr Glu Ile Gln Met Ser Ser Gly Asn Leu Leu Phe Thr Gly His
        435                 440                 445
Leu Lys Cys Arg Leu Arg Met Asp Lys Leu Gln Leu Lys Gly Met Ser
    450                 455                 460
Tyr Ser Met Cys Thr Gly Lys Phe Lys Val Val Lys Glu Ile Ala Glu
465                 470                 475                 480
Thr Gln His Gly Thr Ile Val Ile Arg Val Gln Tyr Glu Gly Asp Gly
                485                 490                 495
Ser Pro Cys Lys Ile Pro Phe Glu Ile Met Asp Leu Glu Lys Arg His
            500                 505                 510
Val Leu Gly Arg Leu Ile Thr Val Asn Pro Ile Val Thr Glu Lys Asp
        515                 520                 525
Ser Pro Val Asn Ile Glu Ala Glu Pro Pro Phe Gly Asp Ser Tyr Ile
    530                 535                 540
Ile Ile Gly Val Glu Pro Gly Gln Leu Lys Leu Asn Trp Phe Lys Lys
545                 550                 555                 560
Gly Ser Ser Ile Gly Gln Met Phe Glu Thr Thr Met Arg Gly Ala Lys
                565                 570                 575
Arg Met Ala Ile Leu Gly Asp Thr Ala Trp Asp Phe Gly Ser Leu Gly
            580                 585                 590
Gly Val Phe Thr Ser Ile Gly Lys Ala Leu His Gln Val Phe Gly Ala
        595                 600                 605
Ile Tyr Gly Ala Ala Phe Ser Gly Val Ser Trp Thr Met Lys Ile Leu
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Ile Gly Val Ile Ile Thr Trp Ile Gly Met Asn Ser Arg Ser Thr Ser
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Val Met Val Gly Ala
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atg tct ggt cgt aaa gct cag gga aaa acc ctg ggc gtc aat atg gta   166
Met Ser Gly Arg Lys Ala Gln Gly Lys Thr Leu Gly Val Asn Met Val
 1               5                  10                  15

cga cga gga gtt cgc tcc ttg tca aac aaa ata aaa caa aaa aca aaa   214
Arg Arg Gly Val Arg Ser Leu Ser Asn Lys Ile Lys Gln Lys Thr Lys
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                20                  25                  30

caa att gga aac aga cct gga cct tca aga ggt gtt caa gga ttt atc   262
Gln Ile Gly Asn Arg Pro Gly Pro Ser Arg Gly Val Gln Gly Phe Ile
        35                  40                  45

ttt ttc ttt ttg ttc aac att ttg act gga aaa aag atc aca gcc cac   310
Phe Phe Phe Leu Phe Asn Ile Leu Thr Gly Lys Lys Ile Thr Ala His
    50                  55                  60

cta aag agg ttg tgg aaa atg ctg gac cca aga caa ggc ttg gct gtt   358
Leu Lys Arg Leu Trp Lys Met Leu Asp Pro Arg Gln Gly Leu Ala Val
65                  70                  75                  80

cta agg aaa gtc aag aga gtg gtg gcc agt ttg atg aga gga ttg tcc   406
Leu Arg Lys Val Lys Arg Val Val Ala Ser Leu Met Arg Gly Leu Ser
                85                  90                  95

tca agg aaa cgc cgt tcc cat gat gtt ctg act gtg caa ttc cta att   454
Ser Arg Lys Arg Arg Ser His Asp Val Leu Thr Val Gln Phe Leu Ile
            100                 105                 110

ttg gga atg ctg ttg atg acg ggt gga atg aag ttg tcg aat ttc cag   502
Leu Gly Met Leu Leu Met Thr Gly Gly Met Lys Leu Ser Asn Phe Gln
        115                 120                 125

ggg aag ctt ttg atg acc atc aac aac acg gac att gca gac gtt atc   550
Gly Lys Leu Leu Met Thr Ile Asn Asn Thr Asp Ile Ala Asp Val Ile
    130                 135                 140

gtg att ccc acc tca aaa gga gag aac aga tgt tgg gtt cgg gca atc   598
Val Ile Pro Thr Ser Lys Gly Glu Asn Arg Cys Trp Val Arg Ala Ile
145                 150                 155                 160

gac gtc ggc tac atg tgt gag gac act atc acg tac gaa tgt cct aag   646
Asp Val Gly Tyr Met Cys Glu Asp Thr Ile Thr Tyr Glu Cys Pro Lys
                165                 170                 175

ctt acc atg ggc aat gat cca gag gat gtg gat tgc tgg tgt gac aac   694
Leu Thr Met Gly Asn Asp Pro Glu Asp Val Asp Cys Trp Cys Asp Asn
            180                 185                 190

caa gaa gtc tac gtc caa tat gga cgg tgc acg cgg acc agg cat tcc   742
Gln Glu Val Tyr Val Gln Tyr Gly Arg Cys Thr Arg Thr Arg His Ser
        195                 200                 205

aag cga agc agg aga tcc gtg tcg gtc caa aca cat ggg gag agt tca   790
Lys Arg Ser Arg Arg Ser Val Ser Val Gln Thr His Gly Glu Ser Ser
    210                 215                 220

cta gtg aat aaa aaa gag gct tgg ctg gat tca acg aaa gcc aca cga   838
Leu Val Asn Lys Lys Glu Ala Trp Leu Asp Ser Thr Lys Ala Thr Arg
225                 230                 235                 240

tat ctc atg aaa act gag aac tgg atc ata agg aat cct ggc tat gct   886
Tyr Leu Met Lys Thr Glu Asn Trp Ile Ile Arg Asn Pro Gly Tyr Ala
                245                 250                 255
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ttc ctg gcg gcg gta ctt ggc tgg atg ctt ggc agt aac aac ggt caa   934
Phe Leu Ala Ala Val Leu Gly Trp Met Leu Gly Ser Asn Asn Gly Gln
            260                 265                 270

cgc gtg gta ttt acc atc ctc ctg ctg ttg gtc gct ccg gct tac agt   982
Arg Val Val Phe Thr Ile Leu Leu Leu Leu Val Ala Pro Ala Tyr Ser
        275                 280                 285

ttt aat tgt ctg gga atg ggc aat cgt gac ttc ata gaa gga gcc agt   1030
Phe Asn Cys Leu Gly Met Gly Asn Arg Asp Phe Ile Glu Gly Ala Ser
    290                 295                 300

ggg gcc act tgg gtg gac ttg gtg cta gaa gga gac agc tgc ttg aca   1078
Gly Ala Thr Trp Val Asp Leu Val Leu Glu Gly Asp Ser Cys Leu Thr
305                 310                 315                 320

atc atg gca aac gac aaa cca aca ttg gac gtc cgc atg att aac atc   1126
Ile Met Ala Asn Asp Lys Pro Thr Leu Asp Val Arg Met Ile Asn Ile
                325                 330                 335

gaa gct agc caa ctt gct gag gtc aga agt tac tgc tat cat gct tca   1174
Glu Ala Ser Gln Leu Ala Glu Val Arg Ser Tyr Cys Tyr His Ala Ser
            340                 345                 350

gtc act gac atc tcg acg gtg gct cgg tgc ccc acg act gga gaa gcc   1222
Val Thr Asp Ile Ser Thr Val Ala Arg Cys Pro Thr Thr Gly Glu Ala
        355                 360                 365

cac aac gag aag cga gct gat agt agc tat gtg tgc aaa caa ggc ttc   1270
His Asn Glu Lys Arg Ala Asp Ser Ser Tyr Val Cys Lys Gln Gly Phe
    370                 375                 380

act gac cgt ggg tgg ggc aac gga tgt gga ttt ttc ggg aag gga agc   1318
Thr Asp Arg Gly Trp Gly Asn Gly Cys Gly Phe Phe Gly Lys Gly Ser
385                 390                 395                 400

att gac aca tgt gca aaa ttc tcc tgc acc agt aaa gcg att ggg aga   1366
Ile Asp Thr Cys Ala Lys Phe Ser Cys Thr Ser Lys Ala Ile Gly Arg
                405                 410                 415

aca atc cag cca gaa aac atc aaa tac aaa gtt ggc att ttt gtg cat   1414
Thr Ile Gln Pro Glu Asn Ile Lys Tyr Lys Val Gly Ile Phe Val His
            420                 425                 430

gga acc acc act tcg gaa aac cat ggg aat tat tca gcg caa gtt ggg   1462
Gly Thr Thr Thr Ser Glu Asn His Gly Asn Tyr Ser Ala Gln Val Gly
        435                 440                 445

gcg tcc cag gcg gca aag ttt aca gta aca ccc aat gct cct tcg gta   1510
Ala Ser Gln Ala Ala Lys Phe Thr Val Thr Pro Asn Ala Pro Ser Val
    450                 455                 460

gcc ctc aaa ctt ggt gac tac gga gaa gtc aca ctg gac tgt gag cca   1558
Ala Leu Lys Leu Gly Asp Tyr Gly Glu Val Thr Leu Asp Cys Glu Pro
465                 470                 475                 480

agg agt gga ctg aac act gaa gcg ttt tac gtc atg acc gtg ggg tca   1606
Arg Ser Gly Leu Asn Thr Glu Ala Phe Tyr Val Met Thr Val Gly Ser
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                485                 490                 495

aag tca ttt ctg gtc cat agg gag tgg ttt cat gac ctc gct ctc ccc   1654
Lys Ser Phe Leu Val His Arg Glu Trp Phe His Asp Leu Ala Leu Pro
            500                 505                 510

tgg acg tcc cct tcg agc aca gcg tgg aga aac aga gaa ctc ctc atg   1702
Trp Thr Ser Pro Ser Ser Thr Ala Trp Arg Asn Arg Glu Leu Leu Met
        515                 520                 525

gaa ttt gaa ggg gcg cac gcc aca aaa cag tcc gtt gtt gct ctt ggg   1750
Glu Phe Glu Gly Ala His Ala Thr Lys Gln Ser Val Val Ala Leu Gly
    530                 535                 540

tca cag gaa gga ggc ctc cat cat gcg ttg gca gga gcc atc gtg gtg   1798
Ser Gln Glu Gly Gly Leu His His Ala Leu Ala Gly Ala Ile Val Val
545                 550                 555                 560

gag tac tca agc tca gtg atg tta aca tca ggc cac ctg aaa tgt agg   1846
Glu Tyr Ser Ser Ser Val Met Leu Thr Ser Gly His Leu Lys Cys Arg
                565                 570                 575

ctg aaa atg gac aaa ctg gct ctg aaa ggc aca acc tat ggc atg tgt   1894
Leu Lys Met Asp Lys Leu Ala Leu Lys Gly Thr Thr Tyr Gly Met Cys
            580                 585                 590

aca gaa aaa ttc tcg ttc gcg aaa aat ccg gtg gac act ggt cac gga   1942
Thr Glu Lys Phe Ser Phe Ala Lys Asn Pro Val Asp Thr Gly His Gly
        595                 600                 605

aca gtt gtc att gaa ctc tcc tac tct ggg agt gat ggc ccc tgc aaa   1990
Thr Val Val Ile Glu Leu Ser Tyr Ser Gly Ser Asp Gly Pro Cys Lys
    610                 615                 620

att ccg att gtt tcc gtt gcg agc ctc aat gac atg acc ccc gtt ggg   2038
Ile Pro Ile Val Ser Val Ala Ser Leu Asn Asp Met Thr Pro Val Gly
625                 630                 635                 640

cgg ctg gtg aca gtg aac ccc ttc gtc gcg act tcc agt gcc aac tca   2086
Arg Leu Val Thr Val Asn Pro Phe Val Ala Thr Ser Ser Ala Asn Ser
                645                 650                 655

aag gtg ctg gtc gag atg gaa ccc ccc ttc gga gac tcc tac atc gta   2134
Lys Val Leu Val Glu Met Glu Pro Pro Phe Gly Asp Ser Tyr Ile Val
            660                 665                 670

gtt gga agg gga gac aag cag atc aac cac cat tgg cac aaa gct gga   2182
Val Gly Arg Gly Asp Lys Gln Ile Asn His His Trp His Lys Ala Gly
        675                 680                 685

agc acg ctg ggc aag gcc ttt tca aca act ttg aag gga gct caa aga   2230
Ser Thr Leu Gly Lys Ala Phe Ser Thr Thr Leu Lys Gly Ala Gln Arg
    690                 695                 700

ctg gca gcg ttg ggc gac aca gcc tgg gac ttt ggc tct att gga ggg   2278
Leu Ala Ala Leu Gly Asp Thr Ala Trp Asp Phe Gly Ser Ile Gly Gly
705                 710                 715                 720
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gtc ttc aac tcc ata gga aga gcc gtt cac caa gtg ttt ggt ggt gcc   2326
Val Phe Asn Ser Ile Gly Arg Ala Val His Gln Val Phe Gly Gly Ala
                725                 730                 735

ttc aga aca ctc ttt ggg gga atg tct tgg atc aca caa ggg cta atg   2374
Phe Arg Thr Leu Phe Gly Gly Met Ser Trp Ile Thr Gln Gly Leu Met
            740                 745                 750

ggt gcc cta ctg ctc tgg atg ggc gtc aac gca cga gac cga tca att   2422
Gly Ala Leu Leu Leu Trp Met Gly Val Asn Ala Arg Asp Arg Ser Ile
        755                 760                 765

gct ttg gcc ttc tta gcc aca gga ggt gtg ctc gtg ttc tta gcg acc   2470
Ala Leu Ala Phe Leu Ala Thr Gly Gly Val Leu Val Phe Leu Ala Thr
    770                 775                 780

aat gtg ggc gcc gat caa gga tgc gcc atc aac ttt ggc aag aga gag   2518
Asn Val Gly Ala Asp Gln Gly Cys Ala Ile Asn Phe Gly Lys Arg Glu
785                 790                 795                 800

ctc aag tgc gga gat ggt atc ttc ata ttt aga gac tct gat gac tgg   2566
Leu Lys Cys Gly Asp Gly Ile Phe Ile Phe Arg Asp Ser Asp Asp Trp
                805                 810                 815

ctg aac aag tac tca tac tat cca gaa gat cct gtg aag ctt gca tca   2614
Leu Asn Lys Tyr Ser Tyr Tyr Pro Glu Asp Pro Val Lys Leu Ala Ser
            820                 825                 830

ata gtg aaa gcc tct ttt gaa gaa ggg aag tgt ggc cta aat tca gtt   2662
Ile Val Lys Ala Ser Phe Glu Glu Gly Lys Cys Gly Leu Asn Ser Val
        835                 840                 845

gac tcc ctt gag cat gag atg tgg aga agc agg gca gat gag atc aat   2710
Asp Ser Leu Glu His Glu Met Trp Arg Ser Arg Ala Asp Glu Ile Asn
    850                 855                 860

gcc att ttt gag gaa aac gag gtg gac att tct gtt gtc gtg cag gat   2758
Ala Ile Phe Glu Glu Asn Glu Val Asp Ile Ser Val Val Val Gln Asp
865                 870                 875                 880

cca aag aat gtt tac cag aga gga act cat cca ttt tcc aga att cgg   2806
Pro Lys Asn Val Tyr Gln Arg Gly Thr His Pro Phe Ser Arg Ile Arg
                885                 890                 895

gat ggt ctg cag tat ggt tgg aag act tgg ggt aag aac ctt gtg ttc   2854
Asp Gly Leu Gln Tyr Gly Trp Lys Thr Trp Gly Lys Asn Leu Val Phe
            900                 905                 910

tcc cca ggg agg aag aat gga agc ttc atc ata gat gga aag tcc agg   2902
Ser Pro Gly Arg Lys Asn Gly Ser Phe Ile Ile Asp Gly Lys Ser Arg
        915                 920                 925

aaa gaa tgc ccg ttt tca aac cgg gtc tgg aat tct ttc cag ata gag   2950
Lys Glu Cys Pro Phe Ser Asn Arg Val Trp Asn Ser Phe Gln Ile Glu
    930                 935                 940

gag ttt ggg acg gga gtg ttc acc aca cgc gtg tac atg gac gca gtc   2998
Glu Phe Gly Thr Gly Val Phe Thr Thr Arg Val Tyr Met Asp Ala Val
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945                 950                 955                 960

ttt gaa tac acc ata gac tgc gat gga tct atc ttg ggt gca gcg gtg   3046
Phe Glu Tyr Thr Ile Asp Cys Asp Gly Ser Ile Leu Gly Ala Ala Val
                965                 970                 975

aac gga aaa aag agt gcc cat ggc tct cca aca ttt tgg atg gga agt   3094
Asn Gly Lys Lys Ser Ala His Gly Ser Pro Thr Phe Trp Met Gly Ser
            980                 985                 990

cat gaa gta aat ggg aca tgg atg atc cac acc ttg gag gca tta gat   3142
His Glu Val Asn Gly Thr Trp Met Ile His Thr Leu Glu Ala Leu Asp
        995                 1000                1005

tac aag gag tgt gag tgg cca ctg aca cat acg att gga aca tca gtt   3190
Tyr Lys Glu Cys Glu Trp Pro Leu Thr His Thr Ile Gly Thr Ser Val
    1010                1015                1020

gaa gag agt gaa atg ttc atg ccg aga tca atc gga ggc cca gtt agc   3238
Glu Glu Ser Glu Met Phe Met Pro Arg Ser Ile Gly Gly Pro Val Ser
1025                1030                1035                1040

tct cac aat cat atc cct gga tac aag gtt cag acg aac gga cct tgg   3286
Ser His Asn His Ile Pro Gly Tyr Lys Val Gln Thr Asn Gly Pro Trp
                1045                1050                1055

atg cag gta cca cta gaa gtg aag aga gaa gct tgc cca ggg act agc   3334
Met Gln Val Pro Leu Glu Val Lys Arg Glu Ala Cys Pro Gly Thr Ser
            1060                1065                1070

gtg atc att gat ggc aac tgt gat gga cgg gga aaa tca acc aga tcc   3382
Val Ile Ile Asp Gly Asn Cys Asp Gly Arg Gly Lys Ser Thr Arg Ser
        1075                1080                1085

acc acg gat agc ggg aaa gtt att cct gaa tgg tgt tgc cgc tcc tgc   3430
Thr Thr Asp Ser Gly Lys Val Ile Pro Glu Trp Cys Cys Arg Ser Cys
    1090                1095                1100

aca atg ccg cct gtg agc ttc cat ggt agt gat ggg tgt tgg tat ccc   3478
Thr Met Pro Pro Val Ser Phe His Gly Ser Asp Gly Cys Trp Tyr Pro
1105                1110                1115                1120

atg gaa att agg cca agg aaa acg cat gaa agc cat ctg gtg cgc tcc   3526
Met Glu Ile Arg Pro Arg Lys Thr His Glu Ser His Leu Val Arg Ser
                1125                1130                1135

tgg gtt aca gct gga gaa ata cat gct gtc cct ttt ggt ttg gtg agc   3574
Trp Val Thr Ala Gly Glu Ile His Ala Val Pro Phe Gly Leu Val Ser
            1140                1145                1150

atg atg ata gca atg gaa gtg gtc cta agg aaa aga cag gga cca aag   3622
Met Met Ile Ala Met Glu Val Val Leu Arg Lys Arg Gln Gly Pro Lys
        1155                1160                1165

caa atg ttg gtt gga gga gta gtg ctc ttg gga gca atg ctg gtc ggg   3670
Gln Met Leu Val Gly Gly Val Val Leu Leu Gly Ala Met Leu Val Gly
    1170                1175                1180
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caa gta act ctc ctt gat ttg ctg aaa ctc aca gtg gct gtg gga ttg   3718
Gln Val Thr Leu Leu Asp Leu Leu Lys Leu Thr Val Ala Val Gly Leu
1185                1190                1195                1200

cat ttc cat gag atg aac aat gga gga gac gcc atg tat atg gcg ttg   3766
His Phe His Glu Met Asn Asn Gly Gly Asp Ala Met Tyr Met Ala Leu
                1205                1210                1215

att gct gcc ttt tca atc aga cca ggg ctg ctc atc ggc ttt ggg ctc   3814
Ile Ala Ala Phe Ser Ile Arg Pro Gly Leu Leu Ile Gly Phe Gly Leu
            1220                1225                1230

agg acc cta tgg agc cct cgg gaa cgc ctt gtg ctg acc cta gga gca   3862
Arg Thr Leu Trp Ser Pro Arg Glu Arg Leu Val Leu Thr Leu Gly Ala
        1235                1240                1245

gcc atg gtg gag att gcc ttg ggt ggc gtg atg ggc ggc ctg tgg aag   3910
Ala Met Val Glu Ile Ala Leu Gly Gly Val Met Gly Gly Leu Trp Lys
    1250                1255                1260

tat cta aat gca gtt tct ctc tgc atc ctg aca ata aat gct gtt gct   3958
Tyr Leu Asn Ala Val Ser Leu Cys Ile Leu Thr Ile Asn Ala Val Ala
1265                1270                1275                1280

tct agg aaa gca tca aat acc atc ttg ccc ctc atg gct ctg ttg aca   4006
Ser Arg Lys Ala Ser Asn Thr Ile Leu Pro Leu Met Ala Leu Leu Thr
                1285                1290                1295

cct gtc act atg gct gag gtg aga ctt gcc gca atg ttc ttt tgt gcc   4054
Pro Val Thr Met Ala Glu Val Arg Leu Ala Ala Met Phe Phe Cys Ala
            1300                1305                1310

atg gtt atc ata ggg gtc ctt cac cag aat ttc aag gac acc tcc atg   4102
Met Val Ile Ile Gly Val Leu His Gln Asn Phe Lys Asp Thr Ser Met
        1315                1320                1325

cag aag act ata cct ctg gtg gcc ctc aca ctc aca tct tac ctg ggc   4150
Gln Lys Thr Ile Pro Leu Val Ala Leu Thr Leu Thr Ser Tyr Leu Gly
    1330                1335                1340

ttg aca caa cct ttt ttg ggc ctg tgt gca ttt ctg gca acc cgc ata   4198
Leu Thr Gln Pro Phe Leu Gly Leu Cys Ala Phe Leu Ala Thr Arg Ile
1345                1350                1355                1360

ttt ggg cga agg agt atc cca gtg aat gag gca ctc gca gca gct ggt   4246
Phe Gly Arg Arg Ser Ile Pro Val Asn Glu Ala Leu Ala Ala Ala Gly
                1365                1370                1375

cta gtg gga gtg ctg gca gga ctg gct ttt cag gag atg gag aac ttc   4294
Leu Val Gly Val Leu Ala Gly Leu Ala Phe Gln Glu Met Glu Asn Phe
            1380                1385                1390

ctt ggt ccg att gca gtt gga gga ctc ctg atg atg ctg gtt agc gtg   4342
Leu Gly Pro Ile Ala Val Gly Gly Leu Leu Met Met Leu Val Ser Val
        1395                1400                1405

gct ggg agg gtg gat ggg cta gag ctc aag aag ctt ggt gaa gtt tca   4390
Ala Gly Arg Val Asp Gly Leu Glu Leu Lys Lys Leu Gly Glu Val Ser
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    1410                1415                1420

tgg gaa gag gag gcg gag atc agc ggg agt tcc gcc cgc tat gat gtg   4438
Trp Glu Glu Glu Ala Glu Ile Ser Gly Ser Ser Ala Arg Tyr Asp Val
1425                1430                1435                1440

gca ctc agt gaa caa ggg gag ttc aag ctg ctt tct gaa gag aaa gtg   4486
Ala Leu Ser Glu Gln Gly Glu Phe Lys Leu Leu Ser Glu Glu Lys Val
                1445                1450                1455

cca tgg gac cag gtt gtg atg acc tcg ctg gcc ttg gtt ggg gct gcc   4534
Pro Trp Asp Gln Val Val Met Thr Ser Leu Ala Leu Val Gly Ala Ala
            1460                1465                1470

ctc cat cca ttt gct ctt ctg ctg gtc ctt gct ggg tgg ctg ttt cat   4582
Leu His Pro Phe Ala Leu Leu Leu Val Leu Ala Gly Trp Leu Phe His
        1475                1480                1485

gtc agg gga gct agg aga agt ggg gat gtc ttg tgg gat att ccc act   4630
Val Arg Gly Ala Arg Arg Ser Gly Asp Val Leu Trp Asp Ile Pro Thr
    1490                1495                1500

cct aag atc atc gag gaa tgt gaa cat ctg gag gat ggg att tat ggc   4678
Pro Lys Ile Ile Glu Glu Cys Glu His Leu Glu Asp Gly Ile Tyr Gly
1505                1510                1515                1520

ata ttc cag tca acc ttc ttg ggg gcc tcc cag cga gga gtg gga gtg   4726
Ile Phe Gln Ser Thr Phe Leu Gly Ala Ser Gln Arg Gly Val Gly Val
                1525                1530                1535

gca cag gga ggg gtg ttc cac aca atg tgg cat gtc aca aga gga gct   4774
Ala Gln Gly Gly Val Phe His Thr Met Trp His Val Thr Arg Gly Ala
            1540                1545                1550

ttc ctt gtc agg aat ggc aag aag ttg att cca tct tgg gct tca gta   4822
Phe Leu Val Arg Asn Gly Lys Lys Leu Ile Pro Ser Trp Ala Ser Val
        1555                1560                1565

aag gaa gac ctt gtc gcc tat ggt ggc tca tgg aag ttg gaa ggc aga   4870
Lys Glu Asp Leu Val Ala Tyr Gly Gly Ser Trp Lys Leu Glu Gly Arg
    1570                1575                1580

tgg gat gga gag gaa gag gtc cag ttg atc gcg gct gtt cca gga aag   4918
Trp Asp Gly Glu Glu Glu Val Gln Leu Ile Ala Ala Val Pro Gly Lys
1585                1590                1595                1600

aac gtg gtc aac gtc cag aca aaa ccg agc ttg ttc aaa gtg agg aat   4966
Asn Val Val Asn Val Gln Thr Lys Pro Ser Leu Phe Lys Val Arg Asn
                1605                1610                1615

ggg gga gaa atc ggg gct gtc gct ctt gac tat ccg agt ggc act tca   5014
Gly Gly Glu Ile Gly Ala Val Ala Leu Asp Tyr Pro Ser Gly Thr Ser
            1620                1625                1630

gga tct cct att gtt aac agg aac gga gag gtg att ggg ctg tac ggc   5062
Gly Ser Pro Ile Val Asn Arg Asn Gly Glu Val Ile Gly Leu Tyr Gly
        1635                1640                1645
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aat ggc atc ctt gtc ggt gac aac tcc ttc gtg tcc gcc ata tcc cag   5110
Asn Gly Ile Leu Val Gly Asp Asn Ser Phe Val Ser Ala Ile Ser Gln
    1650                1655                1660

act gag gtg aag gaa gaa gga aag gag gag ctc caa gag atc ccg aca   5158
Thr Glu Val Lys Glu Glu Gly Lys Glu Glu Leu Gln Glu Ile Pro Thr
1665                1670                1675                1680

atg cta aag aaa gga atg aca act gtc ctt gat ttt cat cct gga gct   5206
Met Leu Lys Lys Gly Met Thr Thr Val Leu Asp Phe His Pro Gly Ala
                1685                1690                1695

ggg aag aca aga cgt ttc ctc cca cag atc ttg gcc gag tgc gca cgg   5254
Gly Lys Thr Arg Arg Phe Leu Pro Gln Ile Leu Ala Glu Cys Ala Arg
            1700                1705                1710

aga cgc ttg cgc act ctt gtg ttg gcc ccc acc agg gtt gtt ctt tct   5302
Arg Arg Leu Arg Thr Leu Val Leu Ala Pro Thr Arg Val Val Leu Ser
        1715                1720                1725

gaa atg aag gag gct ttt cac ggc ctg gac gtg aaa ttc cac aca cag   5350
Glu Met Lys Glu Ala Phe His Gly Leu Asp Val Lys Phe His Thr Gln
    1730                1735                1740

gct ttt tcc gct cac ggc agc ggg aga gaa gtc att gat gcc atg tgc   5398
Ala Phe Ser Ala His Gly Ser Gly Arg Glu Val Ile Asp Ala Met Cys
1745                1750                1755                1760

cat gcc acc cta act tac agg atg ttg gaa cca act agg gtt gtt aac   5446
His Ala Thr Leu Thr Tyr Arg Met Leu Glu Pro Thr Arg Val Val Asn
                1765                1770                1775

tgg gaa gtg atc att atg gat gaa gcc cat ttt ttg gat cca gct agc   5494
Trp Glu Val Ile Ile Met Asp Glu Ala His Phe Leu Asp Pro Ala Ser
            1780                1785                1790

ata gcc gct aga ggt tgg gca gcg cac aga gct agg gca aat gaa agt   5542
Ile Ala Ala Arg Gly Trp Ala Ala His Arg Ala Arg Ala Asn Glu Ser
        1795                1800                1805

gca aca atc ttg atg aca gcc aca ccg cct ggg act agt gat gaa ttt   5590
Ala Thr Ile Leu Met Thr Ala Thr Pro Pro Gly Thr Ser Asp Glu Phe
    1810                1815                1820

cca cat tca aat ggt gaa ata gaa gat gtt caa acg gac ata ccc agt   5638
Pro His Ser Asn Gly Glu Ile Glu Asp Val Gln Thr Asp Ile Pro Ser
1825                1830                1835                1840

gag ccc tgg aac aca ggg cat gac tgg atc ctg gct gac aaa agg ccc   5686
Glu Pro Trp Asn Thr Gly His Asp Trp Ile Leu Ala Asp Lys Arg Pro
                1845                1850                1855

acg gca tgg ttc ctt cca tcc atc aga gct gca aat gtc atg gct gcc   5734
Thr Ala Trp Phe Leu Pro Ser Ile Arg Ala Ala Asn Val Met Ala Ala
            1860                1865                1870

tct ttg cgt aag gct gga aag agt gtg gtg gtc ctg aac agg aaa acc   5782
Ser Leu Arg Lys Ala Gly Lys Ser Val Val Val Leu Asn Arg Lys Thr
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        1875                1880                1885

ttt gag aga gaa tac ccc acg ata aag cag aag aaa cct gac ttt ata   5830
Phe Glu Arg Glu Tyr Pro Thr Ile Lys Gln Lys Lys Pro Asp Phe Ile
    1890                1895                1900

ttg gcc act gac ata gct gaa atg gga gcc aac ctt tgc gtg gag cga   5878
Leu Ala Thr Asp Ile Ala Glu Met Gly Ala Asn Leu Cys Val Glu Arg
1905                1910                1915                1920

gtg ctg gat tgc agg acg gct ttt aag cct gtg ctt gtg gat gaa ggg   5926
Val Leu Asp Cys Arg Thr Ala Phe Lys Pro Val Leu Val Asp Glu Gly
                1925                1930                1935

agg aag gtg gca ata aaa ggg cca ctt cgt atc tcc gca tcc tct gct   5974
Arg Lys Val Ala Ile Lys Gly Pro Leu Arg Ile Ser Ala Ser Ser Ala
            1940                1945                1950

gct caa agg agg ggg cgc att ggg aga aat ccc aac aga gat gga gac   6022
Ala Gln Arg Arg Gly Arg Ile Gly Arg Asn Pro Asn Arg Asp Gly Asp
        1955                1960                1965

tca tac tac tat tct gag cct aca agt gaa aat aat gcc cac cac gtc   6070
Ser Tyr Tyr Tyr Ser Glu Pro Thr Ser Glu Asn Asn Ala His His Val
    1970                1975                1980

tgc tgg ttg gag gcc tca atg ctc ttg gac aac atg gag gtg agg ggt   6118
Cys Trp Leu Glu Ala Ser Met Leu Leu Asp Asn Met Glu Val Arg Gly
1985                1990                1995                2000

gga atg gtc gcc cca ctc tat ggc gtt gaa gga act aaa aca cca gtt   6166
Gly Met Val Ala Pro Leu Tyr Gly Val Glu Gly Thr Lys Thr Pro Val
                2005                2010                2015

tcc cct ggt gaa atg aga ctg agg gat gac cag agg aaa gtc ttc aga   6214
Ser Pro Gly Glu Met Arg Leu Arg Asp Asp Gln Arg Lys Val Phe Arg
            2020                2025                2030

gaa cta gtg agg aat tgt gac ctg ccc gtt tgg ctt tcg tgg caa gtg   6262
Glu Leu Val Arg Asn Cys Asp Leu Pro Val Trp Leu Ser Trp Gln Val
        2035                2040                2045

gcc aag gct ggt ttg aag acg aat gat cgt aag tgg tgt ttt gaa ggc   6310
Ala Lys Ala Gly Leu Lys Thr Asn Asp Arg Lys Trp Cys Phe Glu Gly
    2050                2055                2060

cct gag gaa cat gag atc ttg aat gac agc ggt gaa aca gtg aag tgc   6358
Pro Glu Glu His Glu Ile Leu Asn Asp Ser Gly Glu Thr Val Lys Cys
2065                2070                2075                2080

agg gct cct gga gga gca aag aag cct ctg cgc cca agg tgg tgt gat   6406
Arg Ala Pro Gly Gly Ala Lys Lys Pro Leu Arg Pro Arg Trp Cys Asp
                2085                2090                2095

gaa agg gtg tca tct gac cag agt gcg ctg tct gaa ttt att aag ttt   6454
Glu Arg Val Ser Ser Asp Gln Ser Ala Leu Ser Glu Phe Ile Lys Phe
            2100                2105                2110
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gct gaa ggt agg agg gga gct gct gaa gtg cta gtt gtg ctg agt gaa   6502
Ala Glu Gly Arg Arg Gly Ala Ala Glu Val Leu Val Val Leu Ser Glu
        2115                2120                2125

ctc cct gat ttc ctg gct aaa aaa ggt gga gag gca atg gat acc atc   6550
Leu Pro Asp Phe Leu Ala Lys Lys Gly Gly Glu Ala Met Asp Thr Ile
    2130                2135                2140

agt gtg ttc ctc cac tct gag gaa ggc tct agg gct tac cgc aat gca   6598
Ser Val Phe Leu His Ser Glu Glu Gly Ser Arg Ala Tyr Arg Asn Ala
2145                2150                2155                2160

cta tca atg atg cct gag gca atg aca ata gtc atg ctg ttt ata ctg   6646
Leu Ser Met Met Pro Glu Ala Met Thr Ile Val Met Leu Phe Ile Leu
                2165                2170                2175

gct gga cta ctg aca tcg gga atg gtc atc ttt ttc atg tct ccc aaa   6694
Ala Gly Leu Leu Thr Ser Gly Met Val Ile Phe Phe Met Ser Pro Lys
            2180                2185                2190

ggc atc agt aga atg tct atg gcg atg ggc aca atg gcc ggc tgt gga   6742
Gly Ile Ser Arg Met Ser Met Ala Met Gly Thr Met Ala Gly Cys Gly
        2195                2200                2205

tat ctc atg ttc ctt gga ggc gtc aaa ccc act cac atc tcc tat gtc   6790
Tyr Leu Met Phe Leu Gly Gly Val Lys Pro Thr His Ile Ser Tyr Val
    2210                2215                2220

atg ctc ata ttc ttt gtc ctg atg gtg gtt gtg atc ccc gag cca ggg   6838
Met Leu Ile Phe Phe Val Leu Met Val Val Val Ile Pro Glu Pro Gly
2225                2230                2235                2240

caa caa agg tcc atc caa gac aac caa gtg gca tac ctc att att ggc   6886
Gln Gln Arg Ser Ile Gln Asp Asn Gln Val Ala Tyr Leu Ile Ile Gly
                2245                2250                2255

atc ctg acg ctg gtt tca gcg gtg gca gcc aac gag cta ggc atg ctg   6934
Ile Leu Thr Leu Val Ser Ala Val Ala Ala Asn Glu Leu Gly Met Leu
            2260                2265                2270

gag aaa acc aaa gag gac ctc ttt ggg aag aag aac tta att cca tct   6982
Glu Lys Thr Lys Glu Asp Leu Phe Gly Lys Lys Asn Leu Ile Pro Ser
        2275                2280                2285

agt gct tca ccc tgg agt tgg ccg gat ctt gac ctg aag cca gga gct   7030
Ser Ala Ser Pro Trp Ser Trp Pro Asp Leu Asp Leu Lys Pro Gly Ala
    2290                2295                2300

gcc tgg aca gtg tac gtt ggc att gtt aca atg ctc tct cca atg ttg   7078
Ala Trp Thr Val Tyr Val Gly Ile Val Thr Met Leu Ser Pro Met Leu
2305                2310                2315                2320

cac cac tgg atc aaa gtc gaa tat ggc aac ctg tct ctg tct gga ata   7126
His His Trp Ile Lys Val Glu Tyr Gly Asn Leu Ser Leu Ser Gly Ile
                2325                2330                2335

gcc cag tca gcc tca gtc ctt tct ttc atg gac aag ggg ata cca ttc   7174
Ala Gln Ser Ala Ser Val Leu Ser Phe Met Asp Lys Gly Ile Pro Phe
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            2340                2345                2350

atg aag atg aat atc tcg gtc ata atg ctg ctg gtc agt ggc tgg aat   7222
Met Lys Met Asn Ile Ser Val Ile Met Leu Leu Val Ser Gly Trp Asn
        2355                2360                2365

tca ata aca gtg atg cct ctg ctc tgt ggc ata ggg tgc gcc atg ctc   7270
Ser Ile Thr Val Met Pro Leu Leu Cys Gly Ile Gly Cys Ala Met Leu
    2370                2375                2380

cac tgg tct ctc att tta cct gga atc aaa gcg cag cag tca aag ctt   7318
His Trp Ser Leu Ile Leu Pro Gly Ile Lys Ala Gln Gln Ser Lys Leu
2385                2390                2395                2400

gca cag aga agg gtg ttc cat ggc gtt gcc aag aac cct gtg gtt gat   7366
Ala Gln Arg Arg Val Phe His Gly Val Ala Lys Asn Pro Val Val Asp
                2405                2410                2415

ggg aat cca aca gtt gac att gag gaa gct cct gaa atg cct gcc ctt   7414
Gly Asn Pro Thr Val Asp Ile Glu Glu Ala Pro Glu Met Pro Ala Leu
            2420                2425                2430

tat gag aag aaa ctg gct cta tat ctc ctt ctt gct ctc agc cta gct   7462
Tyr Glu Lys Lys Leu Ala Leu Tyr Leu Leu Leu Ala Leu Ser Leu Ala
        2435                2440                2445

tct gtt gcc atg tgc aga acg ccc ttt tca ttg gct gaa ggc att gtc   7510
Ser Val Ala Met Cys Arg Thr Pro Phe Ser Leu Ala Glu Gly Ile Val
    2450                2455                2460

cta gca tca gct gcc tta ggg ccg ctc ata gag gga aac acc agc ctt   7558
Leu Ala Ser Ala Ala Leu Gly Pro Leu Ile Glu Gly Asn Thr Ser Leu
2465                2470                2475                2480

ctt tgg aat gga ccc atg gct gtc tcc atg aca gga gtc atg agg ggg   7606
Leu Trp Asn Gly Pro Met Ala Val Ser Met Thr Gly Val Met Arg Gly
                2485                2490                2495

aat cac tat gct ttt gtg gga gtc atg tac aat cta tgg aag atg aaa   7654
Asn His Tyr Ala Phe Val Gly Val Met Tyr Asn Leu Trp Lys Met Lys
            2500                2505                2510

act gga cgc cgg ggg agc gcg aat gga aaa act ttg ggt gaa gtc tgg   7702
Thr Gly Arg Arg Gly Ser Ala Asn Gly Lys Thr Leu Gly Glu Val Trp
        2515                2520                2525

aag agg gaa ctg aat ctg ttg gac aag cga cag ttt gag ttg tat aaa   7750
Lys Arg Glu Leu Asn Leu Leu Asp Lys Arg Gln Phe Glu Leu Tyr Lys
    2530                2535                2540

agg acc gac att gtg gag gtg gat cgt gat acg gca cgc agg cat ttg   7798
Arg Thr Asp Ile Val Glu Val Asp Arg Asp Thr Ala Arg Arg His Leu
2545                2550                2555                2560

gcc gaa ggg aag gtg gac acc ggg gtg gcg gtc tcc agg ggg acc gca   7846
Ala Glu Gly Lys Val Asp Thr Gly Val Ala Val Ser Arg Gly Thr Ala
                2565                2570                2575
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aag tta agg tgg ttc cat gag cgt ggc tat gtc aag ctg gaa ggt agg   7894
Lys Leu Arg Trp Phe His Glu Arg Gly Tyr Val Lys Leu Glu Gly Arg
            2580                2585                2590

gtg att gac ctg ggg tgt ggc cgc gga ggc tgg tgt tac tac gct gct   7942
Val Ile Asp Leu Gly Cys Gly Arg Gly Gly Trp Cys Tyr Tyr Ala Ala
        2595                2600                2605

gcg caa aag gaa gtg agt ggg gtc aaa gga ttt act ctt gga aga gac   7990
Ala Gln Lys Glu Val Ser Gly Val Lys Gly Phe Thr Leu Gly Arg Asp
    2610                2615                2620

ggc cat gag aaa ccc atg aat gtg caa agt ctg gga tgg aac atc atc   8038
Gly His Glu Lys Pro Met Asn Val Gln Ser Leu Gly Trp Asn Ile Ile
2625                2630                2635                2640

acc ttc aag gac aaa act gat atc cac cgc cta gaa cca gtg aaa tgt   8086
Thr Phe Lys Asp Lys Thr Asp Ile His Arg Leu Glu Pro Val Lys Cys
                2645                2650                2655

gac acc ctt ttg tgt gac att gga gag tca tca tcg tca tcg gtc aca   8134
Asp Thr Leu Leu Cys Asp Ile Gly Glu Ser Ser Ser Ser Ser Val Thr
            2660                2665                2670

gag ggg gaa agg acc gtg aga gtt ctt gat act gta gaa aaa tgg ctg   8182
Glu Gly Glu Arg Thr Val Arg Val Leu Asp Thr Val Glu Lys Trp Leu
        2675                2680                2685

gct tgt ggg gtt gac aac ttc tgt gtg aag gtg tta gct cca tac atg   8230
Ala Cys Gly Val Asp Asn Phe Cys Val Lys Val Leu Ala Pro Tyr Met
    2690                2695                2700

cca gat gtt ctt gag aaa ctg gaa ttg ctc caa agg agg ttt ggc gga   8278
Pro Asp Val Leu Glu Lys Leu Glu Leu Leu Gln Arg Arg Phe Gly Gly
2705                2710                2715                2720

aca gtg atc agg aac cct ctc tcc agg aat tcc act cat gaa atg tac   8326
Thr Val Ile Arg Asn Pro Leu Ser Arg Asn Ser Thr His Glu Met Tyr
                2725                2730                2735

tac gtg tct gga gcc cgc agc aat gtc aca ttt act gtg aac caa aca   8374
Tyr Val Ser Gly Ala Arg Ser Asn Val Thr Phe Thr Val Asn Gln Thr
            2740                2745                2750

tcc cgc ctc ctg atg agg aga atg agg cgt cca act gga aaa gtg acc   8422
Ser Arg Leu Leu Met Arg Arg Met Arg Arg Pro Thr Gly Lys Val Thr
        2755                2760                2765

ctg gag gct gac gtc atc ctc cca att ggg aca cgc agt gtt gag aca   8470
Leu Glu Ala Asp Val Ile Leu Pro Ile Gly Thr Arg Ser Val Glu Thr
    2770                2775                2780

gac aag gga ccc ctg gac aaa gag gcc ata gaa gaa agg gtt gag agg   8518
Asp Lys Gly Pro Leu Asp Lys Glu Ala Ile Glu Glu Arg Val Glu Arg
2785                2790                2795                2800

ata aaa tct gag tac atg acc tct tgg ttt tat gac aat gac aac ccc   8566
Ile Lys Ser Glu Tyr Met Thr Ser Trp Phe Tyr Asp Asn Asp Asn Pro
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                2805                2810                2815

tac agg acc tgg cac tac tgt ggc tcc tat gtc aca aaa acc tcc gga   8614
Tyr Arg Thr Trp His Tyr Cys Gly Ser Tyr Val Thr Lys Thr Ser Gly
            2820                2825                2830

agt gcg gcg agc atg gta aat ggt gtt att aaa att ctg aca tat cca   8662
Ser Ala Ala Ser Met Val Asn Gly Val Ile Lys Ile Leu Thr Tyr Pro
        2835                2840                2845

tgg gac agg ata gag gag gtc aca aga atg gca atg act gac aca acc   8710
Trp Asp Arg Ile Glu Glu Val Thr Arg Met Ala Met Thr Asp Thr Thr
    2850                2855                2860

cct ttt gga cag caa aga gtg ttt aaa gaa aaa gtt gac acc aga gca   8758
Pro Phe Gly Gln Gln Arg Val Phe Lys Glu Lys Val Asp Thr Arg Ala
2865                2870                2875                2880

aag gat cca cca gcg gga act agg aag atc atg aaa gtt gtc aac agg   8806
Lys Asp Pro Pro Ala Gly Thr Arg Lys Ile Met Lys Val Val Asn Arg
                2885                2890                2895

tgg ctg ttc cgc cac ctg gcc aga gaa aag aac ccc aga ctg tgc aca   8854
Trp Leu Phe Arg His Leu Ala Arg Glu Lys Asn Pro Arg Leu Cys Thr
            2900                2905                2910

aag gaa gaa ttt att gca aaa gtc cga agt cat gca gcc att gga gct   8902
Lys Glu Glu Phe Ile Ala Lys Val Arg Ser His Ala Ala Ile Gly Ala
        2915                2920                2925

tac ctg gaa gaa caa gaa cag tgg aag act gcc aat gag gct gtc caa   8950
Tyr Leu Glu Glu Gln Glu Gln Trp Lys Thr Ala Asn Glu Ala Val Gln
    2930                2935                2940

gac cca aag ttc tgg gaa ctg gtg gat gaa gaa agg aag ctg cac caa   8998
Asp Pro Lys Phe Trp Glu Leu Val Asp Glu Glu Arg Lys Leu His Gln
2945                2950                2955                2960

caa ggc agg tgt cgg act tgt gtg tac aac atg atg ggg aaa aga gag   9046
Gln Gly Arg Cys Arg Thr Cys Val Tyr Asn Met Met Gly Lys Arg Glu
                2965                2970                2975

aag aag ctg tca gag ttt ggg aaa gca aag gga agc cgt gcc ata tgg   9094
Lys Lys Leu Ser Glu Phe Gly Lys Ala Lys Gly Ser Arg Ala Ile Trp
            2980                2985                2990

tat atg tgg ctg gga gcg cgg tat ctt gag ttt gag gcc ctg gga ttc   9142
Tyr Met Trp Leu Gly Ala Arg Tyr Leu Glu Phe Glu Ala Leu Gly Phe
        2995                3000                3005

ctg aat gag gac cat tgg gct tcc agg gaa aac tca gga gga gga gtg   9190
Leu Asn Glu Asp His Trp Ala Ser Arg Glu Asn Ser Gly Gly Gly Val
    3010                3015                3020

gaa ggc att ggc tta caa tac cta gga tat gtg atc aga gac ctg gct   9238
Glu Gly Ile Gly Leu Gln Tyr Leu Gly Tyr Val Ile Arg Asp Leu Ala
3025                3030                3035                3040
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gca atg gat ggt ggt gga ttc tac gcg gat gac acc gct gga tgg gac   9286
Ala Met Asp Gly Gly Gly Phe Tyr Ala Asp Asp Thr Ala Gly Trp Asp
                3045                3050                3055

acg cgc atc aca gag gca gac ctt gat gat gaa cag gag atc ttg aac   9334
Thr Arg Ile Thr Glu Ala Asp Leu Asp Asp Glu Gln Glu Ile Leu Asn
            3060                3065                3070

tac atg agc cca cat cac aaa aaa ctg gca caa gca gtg atg gaa atg   9382
Tyr Met Ser Pro His His Lys Lys Leu Ala Gln Ala Val Met Glu Met
        3075                3080                3085

aca tac aag aac aaa gtg gtg aaa gtg ttg aga cca gcc cca gga ggg   9430
Thr Tyr Lys Asn Lys Val Val Lys Val Leu Arg Pro Ala Pro Gly Gly
    3090                3095                3100

aaa gcc tac atg gat gtc ata agt cga cga gac cag aga gga tcc ggg   9478
Lys Ala Tyr Met Asp Val Ile Ser Arg Arg Asp Gln Arg Gly Ser Gly
3105                3110                3115                3120

cag gta gtg act tat gct ctg aac acc atc acc aac ttg aaa gtc caa   9526
Gln Val Val Thr Tyr Ala Leu Asn Thr Ile Thr Asn Leu Lys Val Gln
                3125                3130                3135

ttg atc aga atg gca gaa gca gag atg gtg ata cat cac caa cat gtt   9574
Leu Ile Arg Met Ala Glu Ala Glu Met Val Ile His His Gln His Val
            3140                3145                3150

caa gat tgt gat gaa tca gtt ctg acc agg ctg gag gca tgg ctc act   9622
Gln Asp Cys Asp Glu Ser Val Leu Thr Arg Leu Glu Ala Trp Leu Thr
        3155                3160                3165

gag cac gga tgt gac aga ctg aag agg atg gcg gtg agt gga gac gac   9670
Glu His Gly Cys Asp Arg Leu Lys Arg Met Ala Val Ser Gly Asp Asp
    3170                3175                3180

tgt gtg gtc cgg ccc atc gat gac agg ttc ggc ctg gcc ctg tcc cat   9718
Cys Val Val Arg Pro Ile Asp Asp Arg Phe Gly Leu Ala Leu Ser His
3185                3190                3195                3200

ctc aac gcc atg tcc aag gtt aga aag gac ata tct gaa tgg cag cca   9766
Leu Asn Ala Met Ser Lys Val Arg Lys Asp Ile Ser Glu Trp Gln Pro
                3205                3210                3215

tca aaa ggg tgg aat gat tgg gag aat gtg ccc ttc tgt tcc cac cac   9814
Ser Lys Gly Trp Asn Asp Trp Glu Asn Val Pro Phe Cys Ser His His
            3220                3225                3230

ttc cat gaa cta cag ctg aag gat ggc agg agg att gtg gtg cct tgc   9862
Phe His Glu Leu Gln Leu Lys Asp Gly Arg Arg Ile Val Val Pro Cys
        3235                3240                3245

cga gaa cag gac gag ctc att ggg aga gga agg gtg tct cca gga aac   9910
Arg Glu Gln Asp Glu Leu Ile Gly Arg Gly Arg Val Ser Pro Gly Asn
    3250                3255                3260

ggc tgg atg atc aag gaa aca gct tgc ctc agc aaa gcc tat gcc aac   9958
Gly Trp Met Ile Lys Glu Thr Ala Cys Leu Ser Lys Ala Tyr Ala Asn
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3265                3270                3275                3280

atg tgg tca ctg atg tat ttt cac aaa agg gac atg agg cta ctg tca   10006
Met Trp Ser Leu Met Tyr Phe His Lys Arg Asp Met Arg Leu Leu Ser
                3285                3290                3295

ttg gct gtt tcc tca gct gtt ccc acc tca tgg gtt cca caa gga cgc   10054
Leu Ala Val Ser Ser Ala Val Pro Thr Ser Trp Val Pro Gln Gly Arg
            3300                3305                3310

aca aca tgg tcg att cat ggg aaa ggg gag tgg atg acc acg gaa gac   10102
Thr Thr Trp Ser Ile His Gly Lys Gly Glu Trp Met Thr Thr Glu Asp
        3315                3320                3325

atg ctt gag gtg tgg aac aga gta tgg ata acc aac aac cca cac atg   10150
Met Leu Glu Val Trp Asn Arg Val Trp Ile Thr Asn Asn Pro His Met
    3330                3335                3340

cag gac aag aca atg gtg aaa aaa tgg aga gat gtc cct tat cta acc   10198
Gln Asp Lys Thr Met Val Lys Lys Trp Arg Asp Val Pro Tyr Leu Thr
3345                3350                3355                3360

aag aga caa gac aag ctg tgc gga tca ctg att gga atg acc aat agg   10246
Lys Arg Gln Asp Lys Leu Cys Gly Ser Leu Ile Gly Met Thr Asn Arg
                3365                3370                3375

gcc acc tgg gcc tcc cac atc cat tta gtc atc cat cgt atc cga acg   10294
Ala Thr Trp Ala Ser His Ile His Leu Val Ile His Arg Ile Arg Thr
            3380                3385                3390

ctg att gga cag gag aaa tac act gac tac cta aca gtc atg gac agg   10342
Leu Ile Gly Gln Glu Lys Tyr Thr Asp Tyr Leu Thr Val Met Asp Arg
        3395                3400                3405

tat tct gtg gat gct gac ctg caa ctg ggt gag ctt atc tgaaacacca    10391
Tyr Ser Val Asp Ala Asp Leu Gln Leu Gly Glu Leu Ile
    3410                3415                3420

tctaacagga ataaccggga tacaaaccac gggtggagaa ccggactccc cacaacctga 10451
aaccgggata taaaccacgg ctggagaacc gggctccgca cttaaaatga aacagaaacc 10511
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gagaccctcc agggaacaaa tagtgggacc atattgacgc cagggaaaga ccggagtggt 10811
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gatgacaaac acaaaaccac t                                           10892
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<210> 53
<211> 3421
<212> PRT
<213> Artificial Sequence

<220>
<223> derived from Yellow Fever virus and Japanese Encephalitis virus

<400> 53

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Met Ser Gly Arg Lys Ala Gln Gly Lys Thr Leu Gly Val Asn Met Val
 1               5                  10                  15
Arg Arg Gly Val Arg Ser Leu Ser Asn Lys Ile Lys Gln Lys Thr Lys
            20                  25                  30
Gln Ile Gly Asn Arg Pro Gly Pro Ser Arg Gly Val Gln Gly Phe Ile
        35                  40                  45
Phe Phe Phe Leu Phe Asn Ile Leu Thr Gly Lys Lys Ile Thr Ala His
    50                  55                  60
Leu Lys Arg Leu Trp Lys Met Leu Asp Pro Arg Gln Gly Leu Ala Val
65                  70                  75                  80
Leu Arg Lys Val Lys Arg Val Val Ala Ser Leu Met Arg Gly Leu Ser
                85                  90                  95
Ser Arg Lys Arg Arg Ser His Asp Val Leu Thr Val Gln Phe Leu Ile
            100                 105                 110
Leu Gly Met Leu Leu Met Thr Gly Gly Met Lys Leu Ser Asn Phe Gln
        115                 120                 125
Gly Lys Leu Leu Met Thr Ile Asn Asn Thr Asp Ile Ala Asp Val Ile
    130                 135                 140
Val Ile Pro Thr Ser Lys Gly Glu Asn Arg Cys Trp Val Arg Ala Ile
145                 150                 155                 160
Asp Val Gly Tyr Met Cys Glu Asp Thr Ile Thr Tyr Glu Cys Pro Lys
                165                 170                 175
Leu Thr Met Gly Asn Asp Pro Glu Asp Val Asp Cys Trp Cys Asp Asn
            180                 185                 190
Gln Glu Val Tyr Val Gln Tyr Gly Arg Cys Thr Arg Thr Arg His Ser
        195                 200                 205
Lys Arg Ser Arg Arg Ser Val Ser Val Gln Thr His Gly Glu Ser Ser
    210                 215                 220
Leu Val Asn Lys Lys Glu Ala Trp Leu Asp Ser Thr Lys Ala Thr Arg
225                 230                 235                 240
Tyr Leu Met Lys Thr Glu Asn Trp Ile Ile Arg Asn Pro Gly Tyr Ala
                245                 250                 255
Phe Leu Ala Ala Val Leu Gly Trp Met Leu Gly Ser Asn Asn Gly Gln
            260                 265                 270
Arg Val Val Phe Thr Ile Leu Leu Leu Leu Val Ala Pro Ala Tyr Ser
        275                 280                 285
Phe Asn Cys Leu Gly Met Gly Asn Arg Asp Phe Ile Glu Gly Ala Ser
    290                 295                 300
Gly Ala Thr Trp Val Asp Leu Val Leu Glu Gly Asp Ser Cys Leu Thr
305                 310                 315                 320
Ile Met Ala Asn Asp Lys Pro Thr Leu Asp Val Arg Met Ile Asn Ile
                325                 330                 335
Glu Ala Ser Gln Leu Ala Glu Val Arg Ser Tyr Cys Tyr His Ala Ser
            340                 345                 350
Val Thr Asp Ile Ser Thr Val Ala Arg Cys Pro Thr Thr Gly Glu Ala
        355                 360                 365
His Asn Glu Lys Arg Ala Asp Ser Ser Tyr Val Cys Lys Gln Gly Phe
    370                 375                 380
Thr Asp Arg Gly Trp Gly Asn Gly Cys Gly Phe Phe Gly Lys Gly Ser
385                 390                 395                 400
Ile Asp Thr Cys Ala Lys Phe Ser Cys Thr Ser Lys Ala Ile Gly Arg
                405                 410                 415
Thr Ile Gln Pro Glu Asn Ile Lys Tyr Lys Val Gly Ile Phe Val His
            420                 425                 430
Gly Thr Thr Thr Ser Glu Asn His Gly Asn Tyr Ser Ala Gln Val Gly
        435                 440                 445
Ala Ser Gln Ala Ala Lys Phe Thr Val Thr Pro Asn Ala Pro Ser Val
    450                 455                 460
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Ala Leu Lys Leu Gly Asp Tyr Gly Glu Val Thr Leu Asp Cys Glu Pro
465                 470                 475                 480
Arg Ser Gly Leu Asn Thr Glu Ala Phe Tyr Val Met Thr Val Gly Ser
                485                 490                 495
Lys Ser Phe Leu Val His Arg Glu Trp Phe His Asp Leu Ala Leu Pro
            500                 505                 510
Trp Thr Ser Pro Ser Ser Thr Ala Trp Arg Asn Arg Glu Leu Leu Met
        515                 520                 525
Glu Phe Glu Gly Ala His Ala Thr Lys Gln Ser Val Val Ala Leu Gly
    530                 535                 540
Ser Gln Glu Gly Gly Leu His His Ala Leu Ala Gly Ala Ile Val Val
545                 550                 555                 560
Glu Tyr Ser Ser Ser Val Met Leu Thr Ser Gly His Leu Lys Cys Arg
                565                 570                 575
Leu Lys Met Asp Lys Leu Ala Leu Lys Gly Thr Thr Tyr Gly Met Cys
            580                 585                 590
Thr Glu Lys Phe Ser Phe Ala Lys Asn Pro Val Asp Thr Gly His Gly
        595                 600                 605
Thr Val Val Ile Glu Leu Ser Tyr Ser Gly Ser Asp Gly Pro Cys Lys
    610                 615                 620
Ile Pro Ile Val Ser Val Ala Ser Leu Asn Asp Met Thr Pro Val Gly
625                 630                 635                 640
Arg Leu Val Thr Val Asn Pro Phe Val Ala Thr Ser Ser Ala Asn Ser
                645                 650                 655
Lys Val Leu Val Glu Met Glu Pro Pro Phe Gly Asp Ser Tyr Ile Val
            660                 665                 670
Val Gly Arg Gly Asp Lys Gln Ile Asn His His Trp His Lys Ala Gly
        675                 680                 685
Ser Thr Leu Gly Lys Ala Phe Ser Thr Thr Leu Lys Gly Ala Gln Arg
    690                 695                 700
Leu Ala Ala Leu Gly Asp Thr Ala Trp Asp Phe Gly Ser Ile Gly Gly
705                 710                 715                 720
Val Phe Asn Ser Ile Gly Arg Ala Val His Gln Val Phe Gly Gly Ala
                725                 730                 735
Phe Arg Thr Leu Phe Gly Gly Met Ser Trp Ile Thr Gln Gly Leu Met
            740                 745                 750
Gly Ala Leu Leu Leu Trp Met Gly Val Asn Ala Arg Asp Arg Ser Ile
        755                 760                 765
Ala Leu Ala Phe Leu Ala Thr Gly Gly Val Leu Val Phe Leu Ala Thr
    770                 775                 780
Asn Val Gly Ala Asp Gln Gly Cys Ala Ile Asn Phe Gly Lys Arg Glu
785                 790                 795                 800
Leu Lys Cys Gly Asp Gly Ile Phe Ile Phe Arg Asp Ser Asp Asp Trp
                805                 810                 815
Leu Asn Lys Tyr Ser Tyr Tyr Pro Glu Asp Pro Val Lys Leu Ala Ser
            820                 825                 830
Ile Val Lys Ala Ser Phe Glu Glu Gly Lys Cys Gly Leu Asn Ser Val
        835                 840                 845
Asp Ser Leu Glu His Glu Met Trp Arg Ser Arg Ala Asp Glu Ile Asn
    850                 855                 860
Ala Ile Phe Glu Glu Asn Glu Val Asp Ile Ser Val Val Val Gln Asp
865                 870                 875                 880
Pro Lys Asn Val Tyr Gln Arg Gly Thr His Pro Phe Ser Arg Ile Arg
                885                 890                 895
Asp Gly Leu Gln Tyr Gly Trp Lys Thr Trp Gly Lys Asn Leu Val Phe
            900                 905                 910
Ser Pro Gly Arg Lys Asn Gly Ser Phe Ile Ile Asp Gly Lys Ser Arg
        915                 920                 925
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Lys Glu Cys Pro Phe Ser Asn Arg Val Trp Asn Ser Phe Gln Ile Glu
    930                 935                 940
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1825 1830 1835 1840
Glu Pro Trp Asn Thr Gly His Asp Trp Ile Leu Ala Asp Lys Arg Pro
1845 1850 1855

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1875 1880 1885
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1925 1930 1935
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1940 1945 1950
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1955 1960 1965
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1970 1975 1980
Cys Trp Leu Glu Ala Ser Met Leu Leu Asp Asn Met Glu Val Arg Gly
1985 1990 1995 2000
Gly Met Val Ala Pro Leu Tyr Gly Val Glu Gly Thr Lys Thr Pro Val
2005 2010 2015
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2275 2280 2285
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<400> 78
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<210> 84

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/32821

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-6

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/32821

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-6, drawn to a chimeric live, infectious, attenuated virus.
Group II, claim(s) 7-12, drawn to a method of using a chimeric, live, infectious, attenuated virus.
Group III, claim(s)13-18, drawn to a nucleic acid molecule encoding a chimeric live, infectious, attenuated virus.
Group IV, claim(s) 19-22, drawn to a method of using a yellow fever virus vector.

and it considers that the International Application does not comply with the requirements of unity of invention (Rules 13.1, 13.2 and 13.3) for the reasons indicated below:

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The invention of group I does not make a contribution over the prior art as evidence by Chambers et al. (WO 98/37911). Since the chimeric, live, infectious, attenuated yellow fever virus is taught in the art as evidence by Chambers et al. (WO 98/37911) the invention lacks unity on the invention as defined by PCT Rule 13.2. The cited reference proves that the technical feature of the Group I does not make a contribution over the prior art, accordingly, the unity of the invention is lacking among all groups.